The roles of pheromones of adult western flower thrips

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Abstract

Frankliniella occidentalis (Pergande) (Thysanoptera: Thripidae) is an invasive worldwide pest of many agricultural, horticultural and ornamental crops. They are difficult to control because of their small size and high resistance to chemical insecticides. The aggregation pheromone of this species is currently used for monitoring, but the full potential for use of this and other pheromones has not yet been explored. Two male-specific headspace volatiles have been previously identified: neryl (*S*)-2-methylbutanoate which acts as an aggregation pheromone and (*R*)-lavandulyl acetate, for which the role is unclear. The roles of these compounds were studied to understand how they can be used in pest management. Laboratory bioassays showed that the aggregation pheromone, apart from being an attractant, also increased the activity level of adult *F. occidentalis*. This could be utilized to activate the thrips out of their concealed spaces within the crop and enhance pickup of chemical insecticides. (*R*)-lavandulyl acetate reduced the walking and take-off activity of adult females but increased the activity level of adult males. The possible role of this compound as a mating pheromone is discussed. The chemical analysis of male-exposed filter paper discs showed the presence of another compound, 7-methyltricosane, which was shown to act as a contact pheromone for species recognition. Adult females respond by raising their abdomen showing mating rejection towards adult males while abdominal wagging sideways was observed in adult males, a behaviour used in aggressive male-male interactions. This is the first identification of a contact pheromone in the order Thysanoptera.

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To God be the glory!

List of species cited

Thysanoptera

Elaphrothrips tuberculatus (Hood, 1908)

Frankliniella intonsa (Trybom, 1895)

Frankliniella occidentalis (Pergande, 1895)

Frankliniella schultzei (Trybom, 1910)

Pezothrips kellyanus (Bagnall, 1916)

Pezothrips dianthi (Priesner, 1921)

Taeniothrips dianthi Priesner, 1921

Thrips fuscipennis Haliday, 1836

Thrips major Uzel, 1895

Thrips palmi Karny, 1925

Thrips tabaci Lindeman, 1889

Non- Thysanoptera

Adalia bipunctata

Amblyseius swirskii

Drosophila spp

Heliothis virescens

Lutzomyia longipalpis

Metarhizium anisopliae

Nauphoeta cinerea

Orius insidious

Thipinema nicklewoodii

Chrysanthemum plant

Dendranthema grandiora Tzvelev

List of abbreviations

ANOVA Analysis of variation

DDT Dichlorodiphenyltrichloroethane

GC Gas Chromatography

GC-MS Gas Chromatography - Mass Spectrometry

GLM General Linear Model

INSV Impatiens Necrotic Spot Virus

IPM Integrated Pest Management

MSDS Material Safety Data Sheet

r intrinsic rate of population increase

SEM Standard Error of the Mean

TSWV Tomato Spotted Wilt Virus

UVA Ultraviolet A

Chapter 1

General Introduction

1.1 Introduction

Thrips are small insects, constituting a single order, the Thysanoptera. About 8000 species are estimated to exist (Lewis, 1997a) of which nearly 5500 species of thrips are described in two suborders: Terebrantia and Tubulifera, consisting of eight families (Mound, 1997, 2005; Morse & Hoddle, 2006). In all thrips, the left mandible is well developed while the right one is resorbed by the embryo (Heming, 1993). Terebrantia species have one propupal and one pupal stage and nearly all lay their eggs inside plant tissue while Tubulifera lay their eggs on the outside of the host plant and have two pupal stages (Morse & Hoddle, 2006).

Several factors have contributed to thrips becoming a serious worldwide pest: small size; high intrinsic rate of natural increase, *r*; high tendency to invade, spread and colonize; and ability to act as vectors of viral plant diseases (Kirk, 1997; Lewis, 1997a; Mound, 1997; Morse & Hoddle, 2006). This results in quarantine risks that negatively affect trans-border trade. Also, human irritation may occur when high numbers of thrips are present, rashes or inflammation in the ears and nose sometimes result from bites causing itching and prickling sensations (Lewis, 1973).

The western flower thrips (*Frankliniella occidentalis* (Pergande)) is one of the most important pests of a wide range of cultivated crops throughout the world (Lewis, 1997a; Kirk & Terry, 2003; Morse & Hoddle, 2006). It is arguably the most studied thrips, accounting for one third of the publications on over 5000 known species of thrips in the

past 30 years (Reitz, 2009). The small size and ability to hide in small spaces within the plant makes their control using chemicals extremely difficult. Therefore, new approaches are needed for effective control and monitoring in glasshouses and in the field. Semiochemicals may offer possible ways to combat the damage caused by thrips.

1.2 Semiochemical responses of thrips

Volatile compounds which mediate behaviour of insects are known as semiochemicals. Allelochemicals and pheromones are two forms of semiochemicals which produce interspecific or intraspecific interactions between insects respectively. Pheromones are volatile or involatile chemical substances that are secreted externally by an individual and induce/initiate a specific reaction in another member of the same species (Karlson & Lüscher, 1959). They are broadly divided into two types: signal pheromones which induce a short term reaction and primer pheromones used for a long-term purpose (Lamprecht *et al.*, 2008). Response to chemical cues by insects makes it possible to locate sources of food and mates (Suckling, 2000). Many studies have been carried out to identify and understand the role of volatile compounds found in many insects (Morgan, 2009; Ray *et al.*, 2009; Hegde *et al.*, 2011; Paschen *et al.*, 2012; Hall *et al.*, 2012). These compounds are categorized according to their function, such as alarm pheromone, sex pheromone, aggregation pheromone, trail pheromone, primer pheromone and many more (Hardie & Minks, 1999).

Plants produce/release semiochemicals that act as attractants or repellants for several pests. These semiochemicals are used naturally by plants to defend against insect attack and interact with the environment. They are referred to as kairomones, allomones and synomones according to the beneficiary of the emitted volatiles. Many studies have reported the role of plant volatiles in plant-insect interactions (Koschier, 2006; Pickett *et al.*, 2006; Shrivastava *et al.*, 2010). Potential control strategies for thrips using different

plant volatiles have been reported by Koschier (2006, 2008). They have been widely reported to serve as attractants, repellents or deterrents. For example, Kirk (1985b) reported that *p*-anisaldehyde attracts *Thrips* species to traps and a similar result was reported by Teulon *et al*. (1993a) when anisaldehyde was added to many kinds of traps. Many plant volatiles have been shown to attract *F. occidentalis* in the laboratory and in the field. Koschier *et al.* (2000) reported that *F. occidentalis* was attracted to flower odours in the chemical classes of benzenoids and monoterpenes. Teulon *et al*. (1999) reported also that *p*-anisaldehyde strongly attracted *F. occidentalis* in a greenhouse trial in New Zealand and in a flight chamber (Davidson *et al.*, 2012). Carvacrol and thymol, constituents of essential oils, were found to cause a reduction in the feeding damage of *F. occidentalis* and a deterrent effect on the selection of oviposition sites by *Thrips tabaci* (Sedy & Koschier, 2003). The effect of these plant volatiles is strongly affected by concentration. Some strong attractants can potentially repel at high concentration (Koschier *et al.*, 2000; Davidson *et al.*, 2008). They are not known to control thrips, but some of these plant volatiles could be used with other strategies to enhance the current biological pest control measures (Koschier, 2008).

Pheromones offer several alternative management techniques for integrated pest management (IPM), such as mass trapping, mating disruption, oviposition deterrence, knock-down enhancement and push-pull strategies (Howse *et al.*, 1998). In categorizing pheromones, their multicomponent nature should be taken into consideration, in which a number of different behaviours may be induced from a single secretion, for example, alarm, mating, aggregation, attraction, arrest (Howse *et al.*, 1998). Hamilton *et al*. (2005) reported the first identification of an aggregation pheromone in the Thysanoptera. Analysis of the male-produced volatile of *F. occidentalis* identified two major components, namely (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate, and five minor components

(Hamilton *et al.*, 2005), however, the neryl (*S*)-2-methylbutanoate has been found to be attractive to both sexes, whereas, the (*R*)-lavandulyl acetate is not attractive and there was no synergy between the two components (Hamilton *et al.*, 2005). One of the major compounds, neryl (*S*)-2-methylbutanoate has been developed into a commercial product and is now widely used for monitoring in the field (Gómez *et al.*, 2006; Broughton & Harrison, 2012).

1.3 Development, variation and distribution

The life cycle of species in the family Thripidae, which includes *F. occidentalis*, consists of an egg, two active feeding larval instars, two inactive non-feeding pupal instars and the adult. Depending on the thrips species and quality of food available, adult females can lay between 30 and 300 eggs in a life time (van Rijn *et al.*, 1995; Lewis, 1997b; Kirk, 1997). In most species, males are derived from unfertilized, haploid eggs, and females are produced from fertlised, diploid eggs (Heming, 1995; Moritz, 1997). The complete life cycle lasts between 10 and 30 days depending on certain factors, most especially, temperature and host plant (Lewis, 1997b). In warm regions there can be up to 12 to 15 generations per year, while there may be only 1 or 2 generations per year in cooler regions (Lewis, 1997b).

Several factors influence the development of *F. occidentalis* such as temperature (Robb, 1989; Katayama, 1997; McDonald *et al.*, 1998; Ishida *et al.*, 2003), host plant (Trichilo & Leigh, 1988; Robb & Parrella, 1991; Gerin *et al.*, 1994; Brødsgaard, 1994a; Gaum *et al.*, 1994; van Rijn *et al.*, 1995; de Kogel *et al.*, 1999), pollen (Hulshof *et al.*, 2003; Zhi *et al.*, 2005) and photoperiod (Brødsgaard, 1994a; Ishida *et al.*, 2003; Whittaker & Kirk, 2004). Robb (1989) reported that development at fluctuating temperatures took a longer period of time compared to a constant temperature of 27°C, however, exposures to low and high temperatures resulted in reduction of oviposition in female *F. occidentalis*,

whereas continuous oviposition was observed at a constant temperature of 27°C throughout the entire life cycle. The rate of development of *F. occidentalis* from egg to adult was found to increase linearly as rearing temperature increased over six temperatures from 10 - 35°C (McDonald *et al.*, 1998). The differences observed in various investigations may be attributed to the cross interaction between temperature and other factors like food quality (Gaum *et al.*, 1994; Soria & Mollema, 1995), photoperiod (Brødsgaard, 1994a; Ishida *et al.*, 2003; Whittaker & Kirk, 2004) and food supplements (Hulshof *et al.*, 2003; Zhi *et al.*, 2005). However, the development of an egg to adult under a favourable temperature of 25 - 30°C can be as short as 9 – 13 d (Robb, 1989; Gaum *et al.*, 1994; Katayama, 1997; Reitz, 2008).

Addition of supplemental foods has been reported to reduce immature development time in the two larval stages and increase female thrips reproductive outputs (Zhi *et al.*, 2005). A similar result was reported by Hulshof *et al*. (2003) that different types of pollen reduced development time and increased fecundity on cucumbers. Nielsen *et al.* (2010) also reported that the difference in intrinsic growth rate observed in two strains of *F. occidentalis* was largely due to the different pollen sources.

De Vries *et al.*, (2004) observed that the effects of gut bacteria on the time of development and oviposition rate depend on the diet available to the thrips. The developmental time was longer and fewer eggs were laid in the thrips with gut bacteria that fed on plant leaves and pollen compared to thrips without gut bacteria. Thrips with gut bacteria that fed on plant leaves alone laid more eggs than thrips without gut bacteria and developed faster.

The effects of these factors should be well understood where possible in the management of *F. occidentalis* development as part of integrated pest management. For

example, the knowledge of development period may assist in determining the time of introduction of biological control agents (Calvo *et al.*, 2012).

F. occidentalis was first reported in 1895 by Pergande in western North America on apricot and potato leaves and thereafter in other states of the USA (Pergande, 1895). *F. occidentalis* is currently found throughout the world. It was first noticed in New Zealand as early as 1934 (Mound & Walker, 1982; Martin & Workman, 1994) and later in the other parts of the world (Kirk & Terry, 2003). Arguably, the spread resulted mainly from the movement of horticultural materials across various locations largely from glasshouses; however, the *F. occidentalis* remained only in western North America for a long time raising a fundamental question of why it was so. The pronounced exposure to insecticides probably also accounted for its spread and extensive establishment in glasshouses (Robb *et al.*, 1988; Brødsgaard, 1989b), because it resulted in high resistance to insecticides (Immaraju *et al.*, 1992; Jensen, 2000; Kiers *et al.*, 2000).

Also, morphological variability is considered to be of high importance in understanding the origin and spread of the *F. occidentalis* (Kirk & Terry, 2003). The field population study conducted by Bryan and Smith (1956) revealed various colour forms associated with adult females. There are three distinct colour forms of adult females namely dark, pale and intermediate, while the adult male is always pale in colour. Bryan and Smith (1956) based their classification on genetics but pale and intermediate colour forms are prominently found and dark forms are rare in glasshouses (Brødsgaard, 1989c; Tommasini & Maini, 1995). Kirk (2002) argued that genetics alone cannot be responsible for the abundance of pale and intermediate colour forms; temperature may play a role in colour variation as observed in *Thrips tabaci* Lindeman (Murai & Toda, 2002). However, a recent study by Rugman-Jones *et al*. (2010) has shown that there are two cryptic species of *F. occidentalis* in its native California. They utilized barcoding to compare the DNA

sequences of nuclear and mitochondrial genes between *F. occidentalis* populations across California. However, it is the form of *F. occidentalis* regarded as the glasshouse strain that has spread across the world and these are the forms in Europe (Rugman-Jones *et al.*, 2010). The glasshouse strain is the one used in this study.

1.4 Reproductive Behaviour

F. occidentalis reproductive behaviour has been studied extensively. The adult males exhibit aggregation behaviour which mainly occurs on flower-heads and sometimes on artificial surfaces (Terry & Schneider, 1993). Choice of flower heads as an aggregation sites may be for food and/or mating. Flower heads are good aggregation sites for mating, feeding and ovipositional activities (Kirk, 1985a; Terry & Gardner, 1990; Terry & Dyreson, 1996; Milne, 1997; Terry, 1997).

There are differences in the duration of interaction and/or fights among male *F. occidentalis* (Terry & Dyreson, 1996). Male *F. occidentalis* when they come across another male, they line up in parallel with their heads pointing in the same direction followed by flicking their abdomens at each other. Aggressive interaction (fight) between a pair of male *F. occidentalis* included rounds of abdominal sparring, and then, one male climbing on the other's thorax and abdomen thereby grabbing his opponent. The opponent would then attempt to free himself by flicking the grabbing male with his abdomen. Repeated rounds of abdominal sparring, grabbing and flicking behaviour between the males occur mainly before one or both males leave the fighting area. A male stayed behind to chase other males which usually resulted in one or both males flicked off the floral lobes or artificial surface (Terry & Dyreson, 1996).

This pattern of behavior among the males involves the flicking of their abdomen when in contact; this mostly occurred at the mating site, which was a flower head, however, the number of thrips present at the site has been shown to affect the level of

activities, the higher the number of males present the lower the level of aggressive behaviour (Kirk, 1985a; Terry, 1995).

Terry & Dyreson (1996) observed that female thrips generally mate with the first male they encountered. This may explain why males remained on the floral corolla lobes where most females landed (Terry, 1997). Furthermore, these behaviours revealed that there are some volatile compounds that are being released to attract females (de Kogel & van Deventer, 2003). Hamilton *et al.*, (2005) confirmed that one of the volatiles, neryl (*S*)-2-methylbutanoate, released by males during this aggregation and fighting attracts both sexes in flight.

1.5 Pest status

F. occidentalis causes severe damage on leaves, flowers and fruits of their host crop. This damage is caused by mechanical action of their mouthparts which involve piercing and sucking out contents of plant cells (Hunter & Ullman, 1989; Harrewijn *et al.*, 1996). The effect of this damage is seen in the form of silvery or necrotic patches on the foliage, flowers and fruits. Damage becomes more pronounced as the foliage, flowers or fruits develop resulting in leaf distortion or petal scarring thus leading to significant losses in the crop yield (Childers, 1997). The scarring of fruits and vegetables has led to the reduction in fruit size and even the total number of fruits. *F. occidentalis* causes indirect damage to major crops; they transmit tospovirus, which causes serious losses in food and fibre production as well as horticultural and ornamental crops (Ullman *et al.*, 1997; Persley *et al.*, 2006). Viruses are spread largely from the transport of infested materials through international trade between the various continents of the world (Latham & Jones, 1997). The transmission of *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus* (INSV) by *F. occidentalis* has been reported by Whitfield *et al.* (2005). *Tomato spotted wilt virus* alone was estimated to cause about $1 billion loss per year worldwide (Goldbach

& Peters, 1994). To transmit this virus, *F. occidentalis* acquires the virus as a first instar or early second instar larva (Wijkamp & Peters, 1993; Tsuda *et al.*, 1996; van de Wetering *et al.*, 1996; Moritz *et al.*, 2004). If adults acquire *Tomato spotted wilt virus* they are not competent in transmission of the virus compared to the larva unless they are derived from infected larvae (De Assis *et al.*, 2004). The ability of *F. occidentalis* to reproduce on a wide range of host plants further complicates the spread of the virus. Adult thrips retain and transmit the virus all year round (Ullman *et al.*, 1993), and the plants they feed on outnumber those that serve as a reproductive host (Paini *et al.*, 2007) as observed in field grown pepper (Brodbeck *et al.*, 2001; Reitz, 2002).

The quantity of virus acquired for transmission by different thrips may be the result of variation in the vector ability of an individual (Sakurai *et al.*, 2004). *F. occidentalis* was found to transmit all four strains of tospovirus in Brazil, *Frankliniella schultzei* transmitted three, while *Thrips palmi* and *Thrips tabaci* did not transmit any form of virus (Nagata *et al.*, 2004). Thrips' ability to transmit tospovirus is dynamic, with some losing this ability (*Thrips tabaci*) while populations of some other species may substantially increase their vector potential (Moritz *et al.*, 2004).

Host plant selection in thrips generally involves several factors. Selection success depends on the plant that can provide mating, feeding and ovipositional sites for the thrips. Terry (1997) stated that landing rates of *F. occidentalis* are affected by a range of plant mediated cues that are important for identification of a potential host. The potential cues included were volatiles and colour. As in many insects of the world, *F. occidentalis* uses colour, shape, size and even scent (Kirk, 1985b; Teulon *et al.*, 1993a, 1999; Terry, 1997) to select their host plants. Some other factors have been suggested to have effects on host plant selection by *F. occidentalis.* A significant effect was reported on the influence of the induction of plant emitted volatiles by herbivores with different feeding habits on selection

of host plant by thrips (Delphia *et al.*, 2007). Feeding by *F. occidenatlis* and *Heliothis virescens* simultaneously produced 11 compounds, and α-humulene and caryophyllene oxide were reported to be the two major compounds. The work suggested that thrips feeding induces volatile responses and that herbivory by insects with different feeding habits can change volatile compound emissions (Delphia *et al.*, 2007).

Floral colour is one of the most important factors in host plant selection by flower thrips and this has been well established by several studies (Kirk, 1984; Yudin *et al.*, 1987; Scott *et al.*, 1989; Brødsgaard, 1989a, c; Gillespie & Vernon, 1990; Teulon & Penman, 1992; Terry, 1997). *F. occidentalis* prefers flowers with blue, low ultra violet reflective white, yellow and blue violet colours. The importance of flowers attracting flower thrips may be connected to the fact that all major activities take place on flowers, as was demonstrated by Kumar *et al.*, (1995) when they reported the lower number of thrips landing on foliage compared to the numbers on flowers. Attraction of *F. occidentalis* to certain flower types may also be due to the scent present in such flowers or perhaps the kind of protection given to larval thrips and the mature adults. There is direct evidence from traps that thrips use floral scent and other plant odours in locating their appropriate host (Kirk, 1985b; Teulon *et al.*, 1993b; Terry, 1997).

Damage caused by *F. occidentalis* makes the use of insecticides a primary strategy for its control (Contreras *et al.*, 2001), especially in virus susceptible crops, where a great number of specific treatments are applied against *F. occidentalis*. The secluded behaviour of *F. occidentalis* protects it from many classes of known chemicals. Many insecticides have been reported to be ineffective in controlling *F. occidentalis* due to the evolution of resistance arising from indiscriminate use of these insecticides (Immaraju *et al.*, 1992; Herron & James, 2005; Bielza, 2008; Zhang *et al.*, 2008; Chena *et al.*, 2011). Jensen (2000) reported that *F. occidentalis* shows resistance to organophosphates, carbamates,

pyrethroids and spinosad and this has been further reported by many researchers as reviewed by Bielza (2008). Insecticide resistance to many chemical groups has been demonstrated among which were: lactone carbametin (Immaraju *et al.*, 1992; Kontsedalov *et al.*, 1998), endosulfan (Brødsgaard, 1994b) and DDT (Zhao *et al.*, 1995). Apart from indiscriminate use of insecticides, short generation time and high fecundity cause resistance to chemicals (Robb *et al.*, 1995; Bielza, 2008; Zhang *et al.*, 2008).

The failure of chlorinated cyclodiene toxaphene to control *F. occidentalis* on cotton in New Mexico in 1961 was the first control failure (Race, 1961). The first study to demonstrate control failure was that of Robb (1989), and since that period, there have been many instances of reduced efficacy of insecticide applications on flower thrips (Robb, 1989). In view of the danger inherent in the indiscriminate use or misuse of insecticides and the damage caused to the environment, underground water and even human health, there is a need to shift the reliance on chemicals to a multifaceted approach in agricultural crop protection; the European Union (EU) has adopted some legislation on pesticide regulation within the member countries, the proposal is to tighten rules on pesticides which will arguably lead to 90% of them being banned (Parente, 2006). Additionally, the EU proposed cut off would ban over 75% of the active ingredients used in pesticides, but decided that new substances would initially be approved for 10 years while those that could be adequately replaced by less toxic active ingredient would require only five years to be approved in order to support and encourage the use of non-chemical alternatives. It is generally accepted that no plant protection products will be allowed to be used within the member countries unless it has been proved and established scientifically that they have no harmful effects on consumers, farmers, local residents and passers by, do not cause unacceptable effects on the environment including ground water and are sufficiently effective against insect pests (Parente, 2006). The pesticides framework directive

2009/128/EC aims to reduce the risks related to the use of pesticides and promote alternative pest management methods.

Control is difficult in agricultural crops infested with *F. occidentalis* necessitating a multidimensional approach in order to have an acceptable degree of control. Effort has been geared towards resolving the inadequacies associated with thrips control, thereby developing a management method involving biological, physical and cultural control combined with low use of some specific chemicals.

Spinosad, a biopesticide has been widely used in integrated pest management because of its novel activity against insects, short half-life, low toxicity to mammals, birds, fish and even to beneficial insects (Thompson *et al.*, 2000). It has been found to be effective in controlling *F. occidentalis* on many agricultural crops suppressing populations of both immature and adult *F. occidentalis* (Cloyd & Sadof, 2000; Jones *et al.*, 2005). However, there are reports that *F. occidentalis* have developed some resistance against spinosad (Bielza *et al.*, 2007; Zhang *et al.*, 2008).

Integrated pest management being a complex management technique must take into consideration the compatibility of all control measures of *F. occidentalis*, understanding the pest, control methods and the interactions between them. It is therefore important to use several available beneficial agents or strategies aimed at different life stages of *F. occidentalis* (Brødsgaard, 2004). Different methods or strategies have been reported in the integrated pest management of *F. occidentalis*: biological control using predatory mites and bugs (Brødsgaard, 2004; Chow *et al.*, 2010), parasitic nematodes (Loomans *et al.*, 1997; Lim & Driesche, 2004), fungi (Butt & Brownbridge, 1997; Ansari *et al.*, 2007) and trap plants (Buitenhuis *et al.*, 2007).

Natural enemies are used in the biological control of *F. occidentalis* both in the field and in glasshouses. These natural enemies include pathogenic nematodes and fungal

pathogens (Butt & Brownbridge, 1997; Loomans *et al.*, 1997; Sabelis & Van Rijn, 1997). Predatory mites control *F. occidentalis* by feeding on the first and second instar larvae (Hessein & Parrella, 1990) although there are some which reside in the soil or growing medium feeding on the pupal stage (Gillespie & Quiring, 1990; Jacobson, 1997). Based on the characteristic features of *F. occidentalis*, biological control of thrips can be critically difficult, ineffective and even more challenging than the use of chemical methods (Parrella, 1995). Therefore, several factors must be considered when implementing biological control programmes to achieve a better and effective result. A situation where a predator feeds on another predator when both are occupying the same habitat should be avoided when using natural enemies for *F. occidentalis* population regulation, the combinations of natural enemies should keep inter-specific interaction among the natural enemies at a minimum level while natural enemy–pest interaction should be optimized (Chow *et al.*, 2010). Similar combinations of predators, parasitoids and pathogens have been reported against leaf miners, aphids, fungus gnats, spider mites, thrips and whiteflies as reviewed by Chow *et al*. (2010).

Chow *et al*. (2010) reported that *Orius insidious* feeds on whichever is the more abundant of *F. occidentalis* and *Amblyseius swirskii* when their numbers were varied on flower and foliage. The time of release of these natural enemies to a large extent can determine how successful a biological control method is for thrips. These natural enemies should be released earlier before the thrips population is well established or else the control programme may not be able to achieve the desired result. It is important to know that eradication or complete regulation of thrips cannot be achieved unless it is on a long-term basis (Jacobson, 1997), therefore, better results can be achieved with long-term crops than those with a short cycle of production (Brødsgaard, 1995).

Thipinema nicklewoodii, a parasitic nematode, has been found to have potential to control *F. occidentalis* (Loomans & Murai, 1997), despite its inability to control the larval thrips population; it was reported by Lim & Driesche (2004) that they control adult *F. occidentalis*, with higher numbers of females being suppressed than male.

The insect pathogenic fungus, *Metarhizium anisopliae* has been studied for the control of major pests of agricultural crops, including *F. occidentalis* (Maniania *et al.*, 2002) and it shows much potential for the control of pests when in the soil (Ansari *et al.*, 2007). The fungus significantly reduced both adult and larval populations of *F. occidentalis*, though the larval control was lower than that of the adults (Maniania *et al.*, 2002). It was further observed that the fungus was highly effective under greenhouse conditions when the thrips population was low to medium (Azaizeh *et al.*, 2002). Ansari *et al.* (2007) observed that *Metarhizium anisopliae* is efficient in suppressing *F. occidentalis* populations in most growing media and is compatible with insecticides.

1.6 Pheromone applications in a *F. occidentalis* control strategy

F. occidentalis alarm pheromone has two components, dodecyl acetate and decyl acetate, produced by larvae in the form of a droplet originating from the anus in response to attack from a predator (Teerling, 1992; Teerling *et al.*, 1993). It was reported that alarm pheromone anal droplet extracts occur in a molar ratio 1.2:1 of dodecyl to decyl and this ratio changes with age (MacDonald *et al.*, 2003). Furthermore, decyl acetate and dodecyl acetate affect both adult and larval movements as demonstrated by walking away from the source (Teerling *et al.*, 1993; MacDonald *et al.*, 2003), decreased landing rate (MacDonald *et al.*, 2002), considerably increased take-off rate (MacDonald *et al.*, 2002) and reduced oviposition rate in adult females (Teerling *et al.*, 1993; Kirk *et al.*, 1999). It was suggested that the alarm pheromone may be involved in causing larvae to retreat to refuges (Venzon *et al.*, 2000) and pre-exposure reduces larval activity which could have an important

bearing if it is used for behavioural manipulation of larvae in glasshouses (Cook *et al.*, 2002). In glasshouse, addition of dodecyl acetate to some insecticides reduces larvae numbers but low effectiveness was recorded for adult mortality (Cook *et al.*, 2002).

Some studies have identified the presence of aggregation pheromone in thrips (Hamilton *et al.*, 2005; Zhang *et al.*, 2011; Zhu *et al.*, 2012). Sex pheromones control a variety of behavioural activities which include alerting, flight, landing, orientation and copulation (Howse *et al.*, 1998). However, only the opposite sex is attracted by sex pheromones whereas aggregation pheromones attract both the male and female of the same species. Adult female *F. schultzei* behaviour was affected when walking or in flight by the odour of adult males of the same species (Milne, 1997; Milne *et al.*, 2002). In *F. occidentalis*, the male-produced pheromone attracts females and males (Kirk & Hamilton, 2004). Thus there is evidence for an attractant role in both *Frankliniella* species. Similar results have been reported for *Frankliniella intonsa* which produces the same compounds as *F. occidentalis* (Zhu *et al.*, 2012). The production site of aggregation pheromone remains unclear. It is established that only adult male *F. occidentalis* produces aggregation pheromone (de Kogel & van Deventer, 2003; Kirk & Hamilton, 2004; Hamilton *et al.*, 2005), therefore the pheromone production gland(s) must be present only in the adult male. The most likely structure is on the abdominal sternites, structures referred to as pore plates (Heming, 1970; El-Ghariani & Kirk, 2008; Mound, 2009; Mound & Masumoto, 2009). *F. occidentalis* had pore plates medially near the anterior edge of abdominal sternites III-VII (El-Ghariani & Kirk, 2008) and this is similar to those in *F. intonsa* (Sudo & Tsutsumi, 2002). This pore plate is thought to be the likely source of the two components in *F. occidentalis*, because the pore plate is associated with subcuticular glandular cells (El-Ghariani & Kirk, 2008). The presence of this structure may also account for the male-produced aggregation pheromone in *F. intonsa* (Lu *et al.*, 2011). Therefore, if the gland is

the source, the presence of pore plates across the genus (Sudo & Tsutsumi, 2002; El-Ghariani & Kirk, 2008) suggests aggregation pheromones are widespread.

In the context of IPM programmes, it is imperative to evaluate pheromones in the control of *F. occidentalis*. There is a need to fully understand the biology and ecology of *F. occidentalis*, in order to establish effective methodology to employ in IPM. Some of the potential application methods are monitoring of the population, mass trapping, mating disruption, lure and infect, lure and kill and push-pull strategy. However, complete eradication of *F. occidentalis* and damage-free crops seems unattainable; therefore the control programme should focus more on use of pheromones to contribute to preventing damage exceeding the economic threshold.

Furthermore, the high cost of registration of pheromones for non-monitoring purposes (Neale, 2000; Chandler, 2003) is another issue that needs to be examined. This may account for the use of pheromones mainly for monitoring. Recent reports indicate that pheromones and some other allelochemicals are effective in monitoring and control of *F. occidentalis* most especially when used with fungi (Niassy *et al.*, 2012).

Pheromones can be used with mass trapping (Lim & Mainali, 2011; Broughton & Harrison, 2012) for monitoring purposes. For pheromones to be used as control measures it has to be registered. The cost of registration is however high. The best trap designs and the cost-effectiveness to farmers must be some of the factors to consider when developing the new technology.

1.7 Aims

It is necessary to identify and develop appropriate new integrated pest management techniques for control of thrips. Pheromones have potential, most especially in the control of *F. occidentalis.* The recent identification of aggregation pheromone in thrips and the status of the pest have made it important to further investigate this pheromone and other

possible pheromones that may play a role in the development of appropriate control technology. Therefore, the proposed aims of this study are:

(1) To investigate the aggregation and mating behaviour of *F. occidentalis* and the potential role of pheromones.

(2) To understand the role of (*R*)-lavandulyl acetate in the biology and ecology of *F. occidentalis*.

(3) To attempt to identify any other male-produced pheromone(s) of *F. occidentalis*.

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Chapter 2

General methods

2.1 Introduction

This chapter describes the methods applicable to more than one chapter of this thesis. Further information specific to each experiment is provided in the chapters where they are reported.

2.2 Rearing of *F. occidentalis*

Different methods have been adapted to rear thrips for specific purposes (Loomans & Murai, 1997). These purposes include screening for pesticide resistance, thrips ecology, behaviour, host-plant interactions, control methods and virus transmission. Thrips can be reared on whole plants, plant parts or artificial substrates. Several factors can influence the choice of rearing medium. For mass rearing of mixed-age thrips, whole plants can be used to obtain the desired population. To produce smaller numbers of even-age thrips, plants parts such as leaves, leaf discs, fruits or pods can be used. Other factors like developmental stage and sex of thrips can also determine the choice of method (Teulon, 1992; Murai & Loomans, 2001). For known-age larvae or adults, bean pods (Bryan & Smith, 1956), flowers (McDonald *et al.*, 1997) and leaf discs (Brødsgaard, 1994) have been used. Because of their minute size and active nature, they must be reared in a container which makes their escape impossible. The rearing container could be cages, plastic rings, Petri dishes or disposable cups, but constant relative humidity must be maintained to prevent condensation within the container. *F. occidentalis* do not usually pupate on host plants,

therefore, an appropriate pupation medium must be provided at the base of the chosen container. Capillary mats, paper and tissue layers have been used as the pupation medium (Bryan & Smith, 1956; McDonald *et al.*, 1997; Dublon, 2009).

2.2.1 Rearing cages

For the purpose of this research, *F. occidentalis* was reared on pot chrysanthemum, *Dendranthema grndiflora* Tzvelev, in six rearing cages. This method, which allows for the rearing of large numbers of mixed-age thrips, has been used at Keele for many years (Kirk & Hamilton, 2004; Hamilton *et al.*, 2005; Dublon, 2009). Mixed-age thrips were reared because of its comparative ease and minimal maintenance time. Also, mixed-age female *F. occidentalis* respond well to aggregation pheromone and not just virgin females (Kirk & Hamilton, 2004). Chrysanthemum plants were introduced into six specially constructed perspex cages (height 600 × width 430 × 430 mm depth). The side walls of each cage were manufactured with transparent Perspex (Rubberfast Ltd., Fenton, UK). A UVA transmissible plastic sheet was used to cover the top of each cage in order to provide more natural `daylight' as it seems more appropriate in the rearing process of *F. occidentalis*. Lighting and temperature are described below. The front wall of each cage (width 370 × 540 mm height) was made into a removable panel to allow easy access. The front panel contained two incorporated vents (84 × 84 mm) to maintain constant relative humidity, the back panel contained a 12 V DC, 0.8W rotating fan (70 × 70 mm), (Papst-Motoren, St. Georgen, Germany) to provide air flow and prevent condensation. The base of the cage was left open, then placed on double layer capillary matting (Vattex Black, Berrycroft Stores Ltd., UK) cut to a slightly larger size than the base of the cage. This was placed into a 525 × 525 mm black plastic tray. This enabled plant watering without opening the cage and also served to provide a moist, but not waterlogged, cage base acting as a potential site for thrips pupation.

2.3 Bioassay conditions

Experiments were carried out in a temperature controlled room. Relative humidity and temperature were constantly monitored with multiple thermo-hygrometers, DRT 880 (Digitron, Torquay, Devon, UK). This room was maintained at a relative humidity that ranged from 70 to 90% and a temperature of 25 ± 2°C. These conditions were selected for their advantages. The temperature of 25 ± 2 º C provides a relatively good egg to egg developmental time of 14 days (van Rijn *et al.*, 1995), longevity and fecundity (Lublinkhof & Foster, 1977).

2.3.1 Lighting

Rearing and bioassays were carried out under a light regime provided by fluorescent tubes (58 W Sylvania Activa 172 professional, 1.5 m length, 240 V AC; Sylvania Lighting International, West Yorkshire, UK in 4 strips,) placed directly above the cages (rearing) and glassware (bioassay). Potted plants and thrips were maintained at a photoperiod of L16:D8. The fluorescent tubes were automatically switched on by 05:00 and off by 21:00 GMT using a timer switch socket. This photoperiod was chosen as long daylength reduces generation period and mortality (Brødsgaard, 1994). The four fluorescent tubes were mounted horizontally on a steel frame at a height of 78 cm above the bench. The light level was measured using a SensorMeter (Philip Harris E30280/1, Philip Harris, Leicestershire, UK) with the tubes providing approximately 1000 lux.

2.3.2 Temperature

The temperature of the room was controlled by an in-built heating system. The fan heater (2 kW, DXC20, Dimplex, UK) was controlled by a thermostat (Allen-Martin, UK) to maintain the desired temperature. Temperature was regularly monitored most especially during the summer when the outside temperature increased considerably. During this

period, the culture room door was left open for some hours to cool it down and no experiment was carried out under such conditions.

2.4 Collection and handling of thrips

Adult *F. occidentalis* were collected from the pot chrysanthemum flowers in the rearing cages. To obtain mixed-age adult males or females, flower heads of the chrysanthemum plants were gently tapped over a white plastic collection dish. Thrips were manually aspirated from the dish using aspirators.

To collect large numbers of *F. occidentalis* a modified aspirator (E713 'Pocket Pooter', Watkins & Doncaster, Kent, UK) was used. It was a standard aspirator modified by adding a blue graduated pipette tip (101 – 1000 µl) (StarLab, Blakelands, Milton Keynes, UK) trimmed to have a 1.5 mm diameter opening in the tip. To prevent thrips being inhaled, a small piece of bridal veil material (fine cotton mesh) was added to the base of the suction tube (John Lewis, Cambridge, UK). A soda glass aspirator vessel (length 50 x 25 mm diam. (Scientific Glass Limited, Hanley, UK)) was used for all the thrips collection. To obtain exposed filter paper discs, 30 mm diam x 20 mm high borosilicate glass (Scientific Glass Laboratories Limited, Tunstall, UK) was used.

When handling a small number of thrips, the thrips were gently transferred with the aid of a deionised water-moistened paintbrush (Aquafine 2 AF62 Flat Shader, Daler-Rowney, UK).

2.5 Cleaning of apparatus

All glassware, such as Petri dishes, glass tubes and aspirator vessels were immersed and cleaned with a brush in 10% Teepol L (Fisher Scientific Supplies, Loughborough, UK) in tap water and rinsed thoroughly with deionised water. Glassware was dried with acetone

(99+ % purity, Fisher Scientific Supplies, Loughborough, UK) and left on a shelf in the laboratory oven at 180°C overnight.

All GC syringes (Hamilton microliter and gastight series, 1 µl, 5 µl, 10 µl, 50 µl, 100 µl, 250 µl, 500 µl and 1000 µl, Fisher Scientific Supplies, Loughborough, UK) were cleaned and rinsed through with hexane (*n*-hexane, for gas chromatography, Fisher Scientific Supplies, Loughborough, UK) prior to and after each use.

2.6 Preparation and storage of chemicals

Synthetic compounds and dilute solutions were stored in accordance with material safety data sheet guidelines. The compounds used were neryl (*S*)-2-methylbutanoate, (*R*)-lavandulyl acetate, (*S*)-lavandulyl acetate, 7-methyltricosane, *n*-tricosane and *n*-hexane. Different concentrations of the synthetic compounds were made through serial dilution from the stock solution. These solutions were stored in sealed glass vials and kept in a freezer. However, before they were used in any experiments, the concentrations were checked by Dr Sudhakar Akella using GC (Chemical Ecology Lab. Keele).

2.7 Data and statistical analysis

All data were analysed using Minitab 16 (Minitab Incorporated, USA). Where parametric analyses were used, Anderson-Darling normality tests were used first to check the residuals for normality. When the residuals were not normally distributed, transformations were undertaken to allow the use of parametric tests. However, where normality could not be achieved by transformation, appropriate non-parametric tests were used. Data were accepted to be statistically significant where $P < 0.05$. Where multiple comparisons were made, Holm's method (Holm, 1979) was used to adjust the *P*-values as

advocated by Wright (1992). These were estimated using WINPEPI (Computer programs for Epidemiologists) version 11.28 (Abramson, 2011).

2.8 References

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Chapter 3

Development of a pheromone bioassay

3.1 Introduction

Behavioural activities leading to non-random spatial distribution and formation of groups are often referred to as aggregation behaviour (Eickwort, 1981). Studies have shown that male adult *F. occidentalis* aggregate on flower heads and occasionally on artificial surfaces (Terry & Schneider, 1993). Flower heads appear to be a good aggregation site for mating and oviposition activities (Kirk, 1985; Terry & Gardner, 1990; Terry & Dyreson, 1996; Terry, 1997). Adult male *F. occidentalis* engage in a fighting behaviour, in which they flick their abdomen when they encounter each other at a mating site. The number of thrips present at the aggregation site affects the activity level (Kirk, 1985; Terry, 1995). It was however observed by Terry & Dyreson (1996) that female thrips mated with the first male they encountered. The rationale behind the competitive fighting among males may be to allow them to mate first when females land at the aggregation site (Terry, 1997). Females reject males for several days after first mating (Terry & Schneider, 1993).

Terry & Gardner (1990) suggested that the aggregation behaviour may be pheromone-mediated. In *F. occidentalis*, these behaviours revealed that some volatile compounds were being released by males that attracted both females and males (Kirk & Hamilton, 2004). Hamilton *et al.*, (2005) reported that two major compounds, neryl (*S*)-2-

methylbutanoate and (*R*)-lavandulyl acetate and some minor compounds were released by adult male *F. occidentalis*, however, it is not known whether these compounds were released only during these aggregation and fighting behaviours.

One of the major compounds, neryl (*S*)-2-methylbutanoate acts as an aggregation pheromone that attracts both sexes while the function of (*R*)-lavandulyl acetate still remains unknown (Hamilton *et al.*, 2005). However, to utilize these pheromonal compounds in population monitoring and control programmes, we need to fully understand their role in the behaviour of *F. occidentalis*. Therefore, it is necessary to carry out well designed bioassays, taking into account the ecological and physiological factors in the laboratory to provide an indication of natural behaviours exhibited in the field. However, it is difficult to design laboratory tests that actually mirror the natural environment.

3.1.1 Bioassays

Bioassays are used to assess the biological activity of behavioural stimuli utilized during host or mate finding and the physiological responses to semiochemicals that influence the quality of host/mate (Hare, 1998). Different bioassay methods have been used (Haynes & Millar, 1998) and many developed over the years with modifications to suit the specific purpose of the experiments (Hare, 1998; Webster *et al.*, 2006; Dublon, 2009). Hare (1998) suggested that laboratory bioassays should consider the behaviour to measure, the experimental design with appropriate statistical analysis method and proper evaluations of the sources of variation.

Generally, bioassays are grouped into those that utilise moving air (olfactometer and wind tunnel) and those that utilise still air (Hare, 1998). The choice of bioassay should largely be determined by the kind of questions to be answered and in a very simple unambiguous manner.

3.1.2 Behavioural responses

In moving-air bioassays, olfactometers are used to measure movement and choice of direction during walking while wind tunnels measure rate of movement and directional choice during flight (Hare, 1998). Still-air bioassays (without air-flow) are mainly utilised for the purpose of measuring behaviour associated with movement and over a short distance from the chemical source. This type of bioassay can demonstrate attraction as an oriented movement towards a chemical source and repellence as an oriented movement away from chemical sources (Dethier, 1960). Care must however be taken to prevent odour saturation within the closed environment and to remove bias against orientation towards the chemical source.

In still-air bioassays, detailed information on turning rates and movements can be achieved by scoring counts. Time-lapse video recording can also be used to provide additional data, most importantly, when groups of individuals are involved. Such recording can provide a basis to track individual changes in their response to a chemical source and to cross-check with the manual scores to provide accurate and unbiased responses from naked eye observations.

3.1.3 Experimental methods: Choice and no-choice test

Studies on preferences (food, host and odour) are conducted as choice or no-choice tests. Choice tests are mostly performed with two sources while multiple choices are performed on at least three sources (e.g. four-arm olfactometer). The use of no-choice or choice tests has been reported widely in relation to host range and specificity (Babendreier *et al.*, 2005; Murray *et al.*, 2010). Depending on the experiments, potentially many factors could affect the results of the chosen test. No-choice tests are convenient for estimation of the widest possible host range (Withers & Mansfield, 2005; Murray *et al.*, 2010) and are also applicable to odour preference experiments giving an opportunity to sample a wide

range of compounds. However, depending on the duration of the experiments, there is a tendency for false positive results. For example, in investigations of host range, a host may eventually be accepted after several rejections, when the preferred host is not available. And this may also be problematic in odour preference studies, where an insect may respond to an odour source after a certain period of time. Therefore, time-dependent changes in behavioural response may be a strong factor in no-choice experiments (Withers & Mansfield, 2005; Murray *et al.*, 2010). No-choice experiments are, however, likely to provide more accurate information than choice experiments because there are no complications that may arise from unknown responses to mixing of host cues (in host range experiments) or saturation of chemicals (in odour preference studies).

Choice experiments are generally expected to show preference more clearly than no-choice experiments. This is due to the fact that time-dependent changes in behavioural response will not occur in the presence of a high ranked host (Van Driesche & Murray, 2004) or preferred odour source (Kirk & Hamilton, 2004). However, mixing of host cues or saturation of chemical source in the experimental arena may greatly affect the outcome of choice experiments. For example, in odour preference studies using a Y-tube olfactometer or petri dish arena, there is a tendency for the mixing of odour. Often times, concentration of the odour or air-flow rate of the Y-tube olfactometer may affect the outcome of such experiments. Some drawbacks have been identified in the statistical analysis of choice experiments (Lockwood, 1998). The two choices are not independent; the insect must accept or respond to one of the choices and reject or not respond to the other. The data is therefore analysed as frequencies or proportions. And because they are not normally distributed, arcsine or square root transformations are employed to rectify truncated tails of data. The data analysis of two choice tests must take into consideration multiple records of an individual by taking an average of such data before it is used for

analysis (Kroon & Housefield, 2003). There can also be the problem of negative autocorrelation in which an individual choosing one half is precluded from choosing the other half (James *et al.*, 2008).

Studies on semiochemical/odour preferences by insects have either used choice (Webster *et al.*, 2006; Carvalho *et al.*, 2011; Cooperband *et al.*, 2012) or no-choice (van Tol *et al.*, 2012) in a moving air flow (Carvalho *et al.*, 2011; Cooperband *et al.*, 2012) or without air flow (Webster *et al.*, 2006; Weeks *et al.*, 2011; van Tol *et al.*, 2012) while some utilised multiple choice (three or more arm olfactometer) (Shamshev *et al.*, 2003). Hare (1998) suggested that bioassay design should consider the complexity of the behaviour to be measured. Therefore, more specialised bioassays should be formulated to measure a specific behaviour instead of a broader assay that attempts to measure a whole range of behavioural process. This may involve modifications of existing and documented bioassays.

3.1.4 Choice filter paper disc bioassay

Olfactometer bioassays of walking adult *F. occidentalis* in the laboratory confirmed the presence of an aggregation pheromone (Kirk & Hamilton, 2004) and this was further demonstrated by the use of synthetic compounds on traps in naturally infested pepper crops (Hamilton *et al.*, 2005). The aggregation pheromone was released into the air from a rubber septum placed on a blue sticky trap and both sexes were attracted, showing the pheromone is volatile. However, Webster *et al.*, (2006) found that Kelly's citrus thrips, *Pezothrips kellyanus* was attracted to filter paper discs exposed to males. It was not clear whether the attracting compound or compounds were released into the air and then absorbed by the filter paper discs or whether the thrips placed the compounds onto the paper discs directly through contact. The attractive properties of filter paper discs exposed to male *P. kellyanus* utilized by Webster et al. (2006) in the laboratory were adapted by

Dublon (2009) to study the behavioural response of *F. occidentalis* to discs exposed to male *F. occidentalis*.

In the filter paper disc bioassays of Dublon (2009), the discs were obtained by exposing them to live thrips using aspirator vessels. This was covered with an initial layer of Parafilm membrane and moisture was provided by placing 100 µl of distilled water on the initial Parafilm. The entire surface was then later covered with another Parafilm layer and the aspirator vessels were placed in the bioassay room at constant temperature. The filter discs were taken out after the exposure period of 24 ± 2 h and were centrally placed on already marked spots in the arena. To observe the test thrips, illumination was provided from below the arena (Petri dish) so that light was from both above and beneath the Petri dish.

Based on the results obtained by Dublon (2009), a potential false positive result was identified. Female *F. occidentalis* were attracted to female-exposed filter paper discs and there is no evidence that adult females produce any compound (Hamilton *et al.*, 2005). This finding led to further study of the bioassay method to identify the sources of variation and to potentially come up with possible solutions. Four weaknesses were observed in the bioassay method of Dublon (2009). The first issue was related to the positive contact result obtained when female-exposed discs were presented to female *F. occidentalis* while the other three were related to the approach and design of the bioassay. The issues and solutions are discussed below:

Issue 1

Parafilm membrane was used to cover the glass exposure tube containing filter paper disc and thrips. Moisture was provided for the exposed thrips by placing water on the initial layer of Parafilm membrane and the whole surface was later

covered with an additional layer of stretched Parafilm. Dublon (2009) observed a response when female thrips were tested against female-exposed filter paper disc which was unexpected based on the previous understanding of adult female *F. occidentalis* (Kirk & Hamilton, 2004; Hamilton *et al.*, 2005). The observed response was possibly due to moisture present on the filter paper disc. The moisture was probably there from the feeding activity of the adult female *F. occidentalis* and this was strongly supported because control filter discs (no-live female thrips exposed) were not moistened. The response to moistened exposed-filter paper disc was not observed with dry non thrips- exposed discs suggesting the response was an artifact caused by the activities of thrips during the exposure period. There is a possibility that thrips piercing the Parafilm with their punch and sucking mouthparts (Moritz, 1997) allowed moisture to leak through and moisten the filter paper. The problem was not apparent with male- exposed filter paper discs because a response to the pheromone was already present. The response observed may be due to thrips seeking out moisture. This issue was identified by Dublon (2009).

Solution to issue 1

To solve this problem, two approaches were used. The first approach was to investigate whether moisture can be completely eliminated during the exposure process. Therefore, a test was done to determine if live thrips can survive without water during the entire period of exposure. The second approach was, if use of water cannot be eliminated, then a better method of water application should be used. Water was applied without touching the disc into the opposite side of the filter paper disc on the base of the glass tubes. This allows for equal weighting of the discs in both treatment and control glass tubes.

Issue 2

Dublon (2009) provided illumination to observe the activities of thrips when on the underside of the filter paper disc by placing the Petri dish on a lightbox (556-272 'Artwork Lightbox', RS Components, Northamptonshire, UK). This was to facilitate the observation of the silhouette of thrips through the filter paper discs. Such an approach does not reflect field conditions as the source of illumination in the field is above and not below. This also increases the temperature which may affect the behaviour of thrips.

Solution to issue 2

To address this issue, a rectangular pane of glass (length 600 mm, width 200 mm, K2 Glass and Glazing Limited, Tunstall, UK) was placed horizontally on a bench in the bioassay room. The glass was supported, 10 cm high on the bench which allows the thrips to be seen and properly observed from above and below.

Issue 3

The filter paper discs were exposed to live thrips for 24 ± 2 h in a constant temperature room maintained at 25 ± 2 ºC. Thrips are known to aggregate during day time (Terry & Gardner, 1990) and it has been shown that adult male *F. occidentalis* produce pheromone during patrolling (Kirk & Hamilton, 2004). The time of exposure seems too long which did not allow for more experiments in a day thereby affecting the efficiency. Also, this period was not within the normal photoperiod of 16:8 light:dark, as continuous light was provided during the exposure period making comparison with the natural field situation difficult. The 24 h photoperiod did not allow the time frame in which the pheromones were being

produced to be detected. The pheromone produced might be less active and thereby not detected by the thrips.

Solution to issue 3

To address this long period of exposure, pilot experiments were done using different time periods. The shortest period that gave a reliable and consistent result, and which allows more experiment to be done was chosen. This time period was then used for exposure process throughout the research project.

Issue 4

Thrips response was measured as the number of contacts thrips made with filter paper discs; this may not truly reflect the strength of the behavioural response of thrips. The contact may be due to hesitation or not making a decision and all the contact may be from a single thrips within one bioassay.

Solution to issue 4

To solve this problem, two other methods were tested alongside the contact method in order to select a better method. The method that gave a clear behavioural response was chosen. The methods were: arena method, disc method and contact method.

Therefore, it is important to improve the Dublon (2009) bioassay to eliminate technical problems, reduce/eliminate ambiguity and measure responses in a simpler way. Such improvements are needed to determine the real response of adult *F. occidentalis* to any detectable volatiles.

3.1.5 Experimental aims

This chapter attempts to develop an improved filter paper disc bioassay method to measure the behavioural responses of *F. occidentalis* to pheromones. This will eliminate artefacts and technical issues associated with the Dublon (2009) bioassay method. This chapter also aims to test whether female *F. occidentalis* produce any detectable volatile.

Whether female *F. occidentalis* produce any detectable volatile is also tested.

3.2 Materials and methods

3.2.1 Pheromone assay

Behavioural responses of *F. occidentalis* to pheromone were determined using different measurement methods with the aim of using a clear and simple method. The bioassays used a 100 mm glass petri dish and this was divided into equal halves of treatment and control. In the "arena method", the response was calculated from the number of thrips within the divided area of the glass petri dish between exposed (treatment) and non-exposed (control) sides. The "contact method" measured the touching of exposed (treatment) and non-exposed (control) filter paper discs by freshly introduced thrips. While in the "disc method", the response was calculated from the number of test thrips on or under the exposed (treatment) and non-exposed (control) filter paper disc.

In the bioassay process, arena, contact and disc methods were used for measuring behavioural response as described above. This was used to determine the best bioassay method to give a clear and simple measurement that will be easy to interpret and explain in the context of behavioural experiments. In arena measurement, the Petri dish was divided into two equal halves using the treatment and control filter discs as the basis of division. The number of adult thrips found on each half of the Petri dish, number of contacts with filter discs and those found on the filter paper discs at successive time periods were

recorded. Records were taken every three minutes for a total of 30 minutes. The number of test thrips in each of the sides were recorded every 3 min for 30 min giving a total of 10 scores; this was averaged to get a single data point. It was later processed to obtain the response index for each Petri dish (see 3.2.5). However, activities of the test thrips were also observed and recorded during the exposure and bioassay process to provide further information on the behavioural responses. For example, what thrips do underneath the filter paper discs and time spent in contact with the discs.

By using the measurement method that gave the clearest behavioural response to pheromone, it was hoped that the behavioural response to exposed discs could be compared with the response to synthetic compounds on filter paper discs (Chapter 4) and on a natural substrate (Chapter 6). If eventually it did not replicate the expected behavioural response, detection of any other possible compounds would then be carried out using chromatographic techniques (Chapter 5).

3.2.2 Exposure: obtaining thrips-exposed filter paper discs

To test the behavioural response of *F. occidentalis* to natural pheromone, filter paper discs were exposed to 15 male or female *F. occidentalis* for 5 hours. The pilot experiment revealed that thrips were able to respond to male-exposed discs after exposure of 5 hours and this was later adopted in order to generate more experimental results within a day. The exposed discs were later used in the pheromone bioassay. In order to expose the discs, cellulose filter discs (Whatman International Limited (1001020) grade 1, 20mm diam.) were gently placed with hexane-cleaned forceps on the base of 20 x 30 mm (height x diam.) borosilicate glass tubes (Scientific Glass Laboratories Limited, Tunstall, UK). Deionised water (15µl) was pipetted (without touching the disc) (Digital 4 – 20µl, FinniPipette, Finland) into the opposite side of the filter paper disc on the base of the borosilicate glass tubes to provide moisture to sustain the thrips (Figure 3.1).

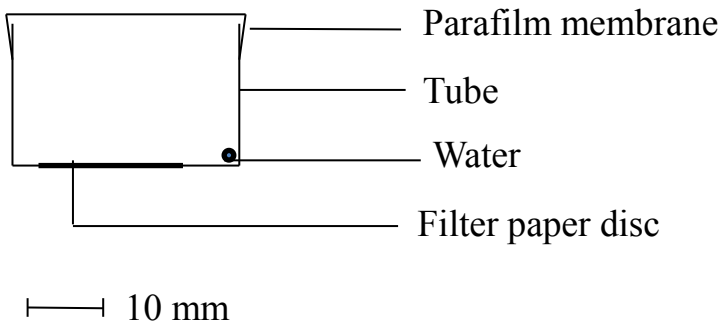

Figure 3.1 Cross section view of a glass tube used in the exposure of filter paper disc to *F. occidentalis*

A specified number of mixed age adult thrips were aspirated from a white dish containing *F. occidentalis* into length 50 x 25 diam. mm soda glass aspirator vessel (Scientific Glass Limited, Hanley, UK). A magnifier head loupe (L111B, Lensel Optics Pvt. Limited, India) was used during the process. Each aspirator vessel was gently removed from the aspirator and the *F. occidentalis* were transferred quickly by gentle tapping of the aspirator vessel into the glass tubes with the filter paper disc. The control was prepared in the same way but left without live thrips. To prevent thrips and volatile from escaping, the glass tubes were covered with a stretched piece of 35 x 50 mm length of Parafilm membrane (Parafilm M, Pechiney Plastic Packaging, WI, USA). In order to observe the test thrips activities from below, the glass tubes (treatment and control) were placed on a horizontal 600 x 200mm rectangular pane of glass (K2 Glass and Glazing Limited, Tunstall, UK) in the bioassay room with a constant temperature of 25°C ± 2°C for 5 hours and illumination was provided throughout by a light rig containing 4 x fluorescent tubes (58 W Sylvania Activa 172 Professional; Sylvania Lighting International, West Yorshire, UK) providing approximately 1000 lux of light. The exposure was carried out in the early hours of the day from 8:30 am – 2:30 pm to reproduce the response recorded during the pilot experiment and for consistency. A typical behavioural response of adult female *F. occidentalis* was

recorded using the video function of a camera (Canon EOS 550D) and was sent to Dr Ian Dublon for digitization.

3.2.3 Filter paper disc: choice bioassay

To determine the behavioural response of test thrips to exposed discs, a Petri dish was used as an observation arena. This was to determine the preferred disc between thrips-exposed discs and no-thrips exposed discs. A set of 100 mm diameter glass Petri dishes and their lids (Anumbra, Scientific Glass Laboratories Limited, Tunstall, UK) were cleaned and prepared as previously described in section 2.5. Two equidistant positions, 20 mm from the centre of the dish to both sides, were marked out on the underside base of the Petri dish in order to position the filter discs without any bias (Figure 3.2). Three Petri dish positions, placed 100 mm apart, were also marked on a rectangular plain glass with equal distance (100 mm) between the marked positions (Figure 3.3). Cellulose filter discs (Whatman International Limited (1001020) grade 1, 20 mm diam.) exposed to thrips and the control, were placed on the two positions marked on the Petri dishes using forceps cleaned with hexane (*n*-hexane, pesticide residue analysis grade (1526764), VWR International Limited, Poole, UK). A specified number of mixed-age adult *F. occidentalis* were aspirated and anaesthetised by exposing them to a gentle 10 s stream of carbon dioxide (British Oxygen Company, UK) and transferred to the middle of the Petri dish containing the exposed disc and the control. The lids were added and a 90 x 20 mm strip of Parafilm membrane was stretched round the edges of the Petri dish covering both the base and the lid in order to create a tight seal against thrips escape. Treatment and control filter disc positions on the Petri dishes were randomly assigned using random numbers within each replicate to reduce any possible directional room bias. This bioassay was carried out after the exposure process between the hours of 02:30 pm – 04:00 pm.

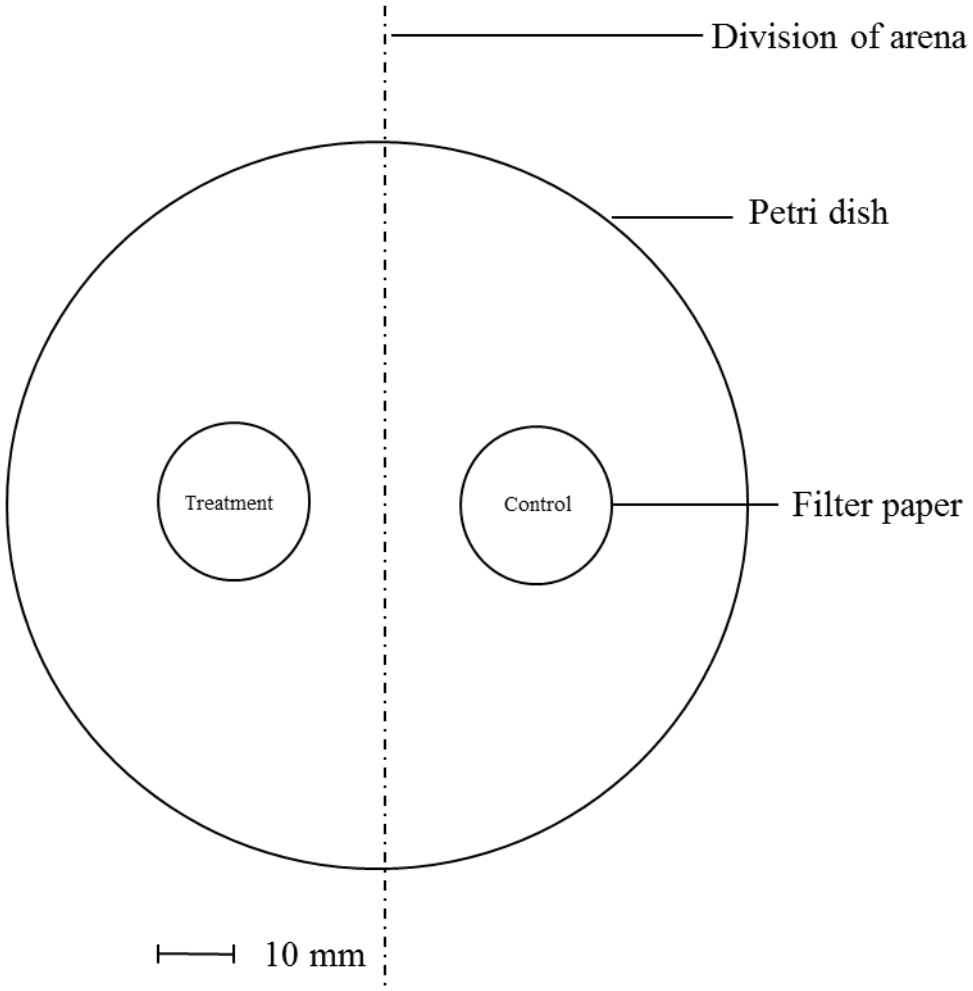

Figure 3.2 Top view of Petri dish arena used in the bioassay of behavioural response of *F. occidentalis* to an exposed disc (treatment) and a non-exposed disc (control)

3.2.3.1 Effect of time of exposure

The response of adult female *F. occidentalis* to filter paper discs exposed to males with varying times of exposure was tested. This was carried out to detect if there is any dose-response effect and behavioural differences from the amount of compounds on the exposed discs. Filter paper discs were exposed to adult male *F. occidentalis* for different duration. The filter paper discs were exposed in a similar way to previously described process (see 3.2.2) and the response bioassay was carried out as described in 3.2.3.

However, this was done in two sets. The first set comprised of ½, 1½ and 2½ h exposure while the other set comprised of 2½, 5 and 7½ h. The exposure process was done in such a way that they all ended at the same time (02:00 pm) which means they were started at different times of the day. To avoid day variation, treatments were completely randomised whereby all treatments were tested each day of the experiment starting from different time of the day but ending at the same time in the afternoon.

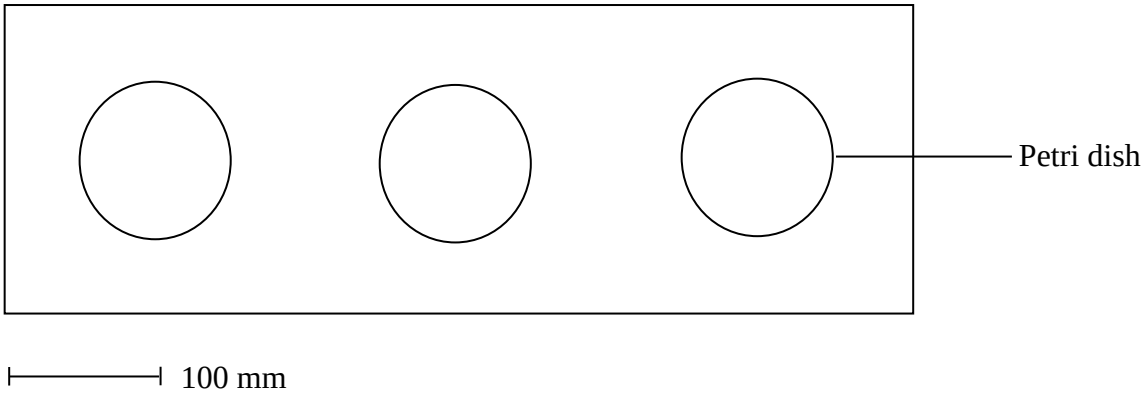

Figure 3.3 Top view of the arrangement of Petri dishes and filter paper discs on a rectangular plain glass 600 x 200 mm (length x width) shelf during the bioassays.

3.2.4 Behaviour and mortality during exposure

During exposure, visual observation was used to distinguish between stationary and dead thrips and a record of dead thrips noted. This was done to determine the mortality rate of both male and female *F. occidentalis* in the presence or absence of moisture. The absence of movement of the legs and antennae was used to identify dead thrips. Numbers of dead thrips were recorded every 30 min during the exposure process to give the survival rate. Movement and position of the adult thrips within the glass tubes were recorded, according to whether they were on the filter disc, beneath the disc, around the wall of the tubes or on the Parafilm membrane. The exposure lasted for 5 hours.

3.2.5 Data processing and statistical analysis

Statistical tests were performed using Minitab for Windows Release 16.1 (Minitab Incorporated, USA). As discussed in section 3.1.3, there are issues with two-choice tests mainly because the data are not always normally distributed and arcsine transformations of such data are usually employed to correct the violated analysis of variance assumptions. The data were processed to produce a response index in which no individuals were omitted from the analyses since all were counted. In some experiments, where there are "no-choice" responders they are usually omitted from analyses giving rise to unequal sample size (Takakura, 2009). However, this experiment did not give room for any "no-choice" responders because all the test thrips were used in the analysis. The test thrips must be in the treatment half (exposed-disc) or control half (blank disc).

The response index was processed using this form$(T - C)/(T + C)$. Response index (RI) is given as:

$$\text{RI} = \frac{\text{T}-\text{C}}{\text{T}+\text{C}} \text{ (Hare, 1998)}$$

All data were tested and confirmed for normality as described in 2.7. Mortality rate was analysed using Kaplan-Meier estimates. Measurement methods data were analysed using ANOVA from the calculated response index value.

Where T is the number of test thrips in the treatment half (exposed-disc) and C is the number of test thrips present in the control half (blank disc) (see 3.3.4). When there is no difference between treatment and control, the response index is zero. The RI ranges from -1 when the treatment half (exposed-disc) is never chosen to +1 when the treatment half (exposed-disc) is always chosen. All test thrips were used in the data analysis and this larger sample size tends to make the data normally distributed as compared with data that has many no-responders. The averaged single data point was based on the same

denominator. For example, 15 thrips were used and recording done every 3 min for 30 min period giving 10 scores. If all 15 thrips were in treatment half throughout the experiment, it means that the total thrips for treatment side is 15 multiply by 10 giving 150 thrips while 0 for control side. The total thrips (T + C) is 150 thrips which is a common denominator throughout.

Furthermore, none of the RI was close to the tail end (+1 and -1) of the data which may also account for the normal distribution. Also, data within the range of 30% to 70% is homogenous and no arc-sine transformation is necessary in such cases (Sokal & Rohlf, 1995). The Response Index was analysed using a one sample *t*-test to detect difference from zero, paired *t*-test where applicable and general linear model ANOVA.

3.3 Results

3.3.1 Survival test

To find a way of providing moisture in the treatment and control discs to prevent artefacts described earlier, survival tests were done. This was designed to investigate whether adult *F. occidentalis* can be exposed to filter paper discs with or without moisture for fewer hours than 24 ± 2h as used previously by Dublon (2009). This is to find out if the exposed thrips can survive without water for a sufficient period of time (hours) thereby eliminating the use of water during the exposure process. This was proposed to determine the source of variation/artefacts recorded in the Dublon (2009) bioassays. But if not, a better method of application of water should be used.

When 15 thrips were exposed without moisture, there was significantly higher mortality rate of adult males and females (*Chi*-square: $n = 75$, $df = 1$, $\chi^2 = 9.17$, $P = 0.011$). At 5 h period of exposure, 45.33% male and 66.67% female survived (Figure 3.4). However, with moisture, no mortality was recorded for either sex over the entire 5 h time period for exposure experiment (*Chi*-square: $n = 75$, $df = 1$, $\chi^2 = 0$, $P = 1.00$) (Figure 3.5).

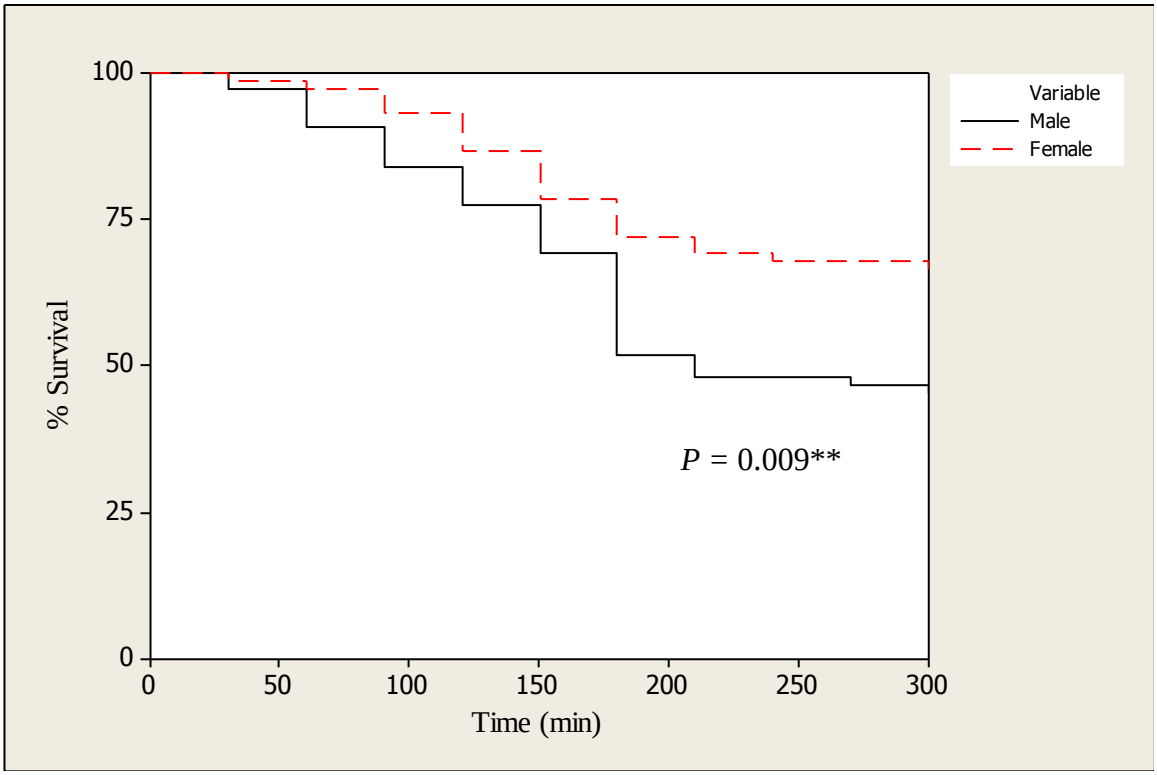

Figure 3.4 Survival of adult male and female *F. occidentalis* exposed to a filter paper disc placed in a glass tube without moisture. Survival curves were calculated for a duration of 300 min. The survival curves of adult male and female were compared using log-rank test statistics. n= 75.

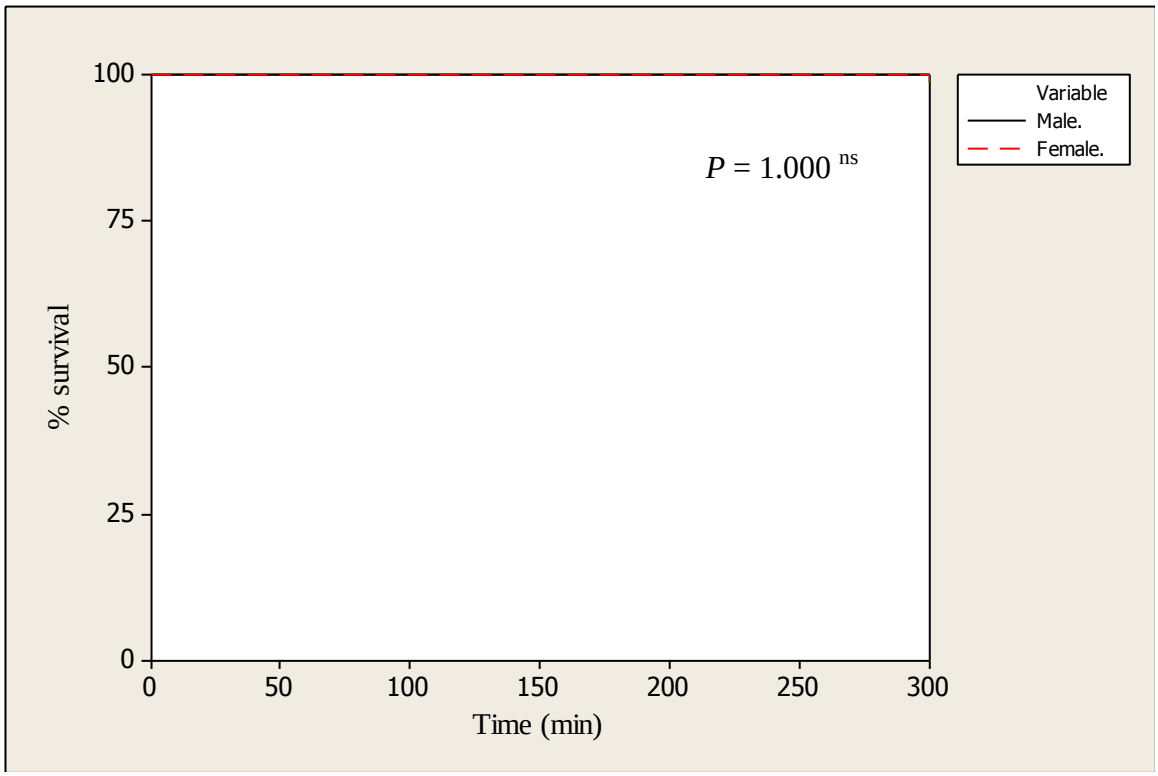

Figure 3.5 Survival of adult male and female *F. occidentalis* exposed to a filter paper disc placed in a glass tube with moisture. Survival curves were calculated for a duration of 300 min. The survival curves of adult male and female were compared using log-rank test statistics. n= 75. This graph is on the same scale as Figure 3.4 for comparison. Both adult male and female thrips survived the 300 min giving a 100% survival rate. n=75.

3.3.2 Percentage time spent in contact with filter paper disc

The time spent by thrips on the filter paper disc during exposure was measured to evaluate whether they were in contact with the disc (on or beneath). This was to shed more light on the activities involved by the thrips during the exposure process and how pheromone might be applied. There was a significance difference between the percentage time spent by adult male and female thrips (ANOVA, $F_{1,23} = 551.75$, $P < 0.0001$). At 5 h

exposure period, 80% of time was spent on the disc by male while 42% time was recorded by female thrips (Figure 3.6).

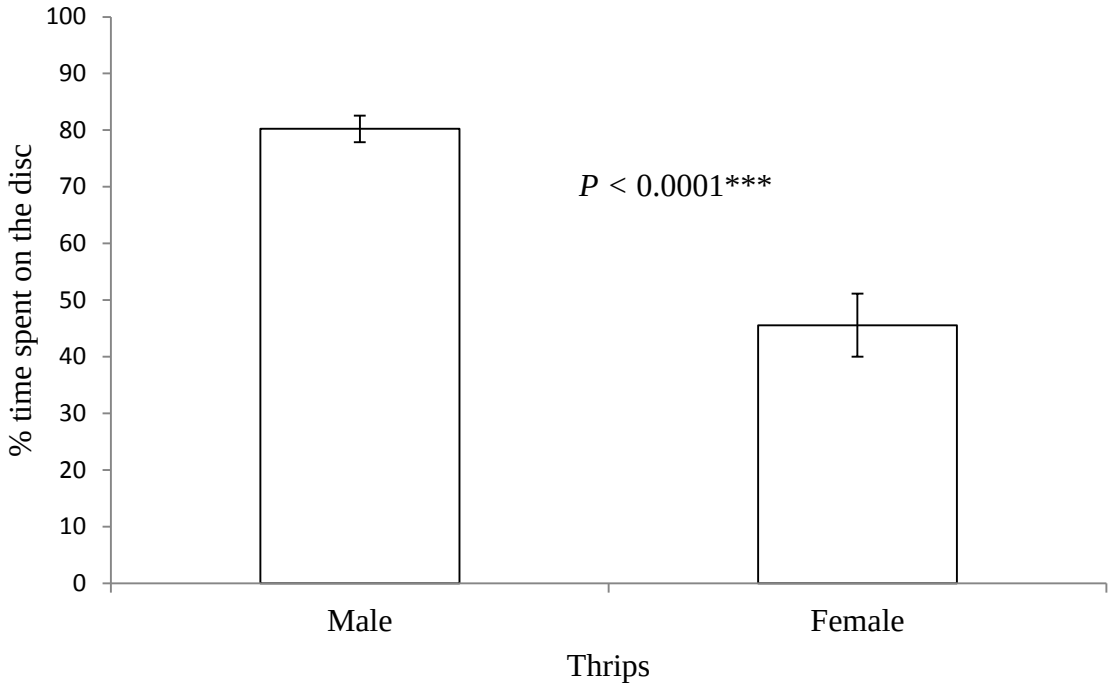

Figure 3.6 The mean (± SE) estimated percentage of time spent by thrips on the filter disc during the exposure period of 300 min. There was a significant difference between the time spent on the disc by male and female *F. occidentalis* (Mann-Whitney test, W= 222.0, P< 0.0001)

3.3.3 Measurement methods

Behavioural response to exposed discs was measured using three methods: 'arena', 'contact' and 'disc' to find the most practical, simple and biologically relevant method. There was no significant difference among the methods (ANOVA, $F_{2,26} = 0.48$, $P = 0.624$). However, the arena method gave the lowest variability while the disc method was highly variable (Figure 3.7). The mean response index for arena, contact and disc methods were 0.59 ± 0.02, 0.47 ± 0.05 and 0.52 ± 0.13 respectively. Thus the arena method appeared to be the most effective and powerful method.

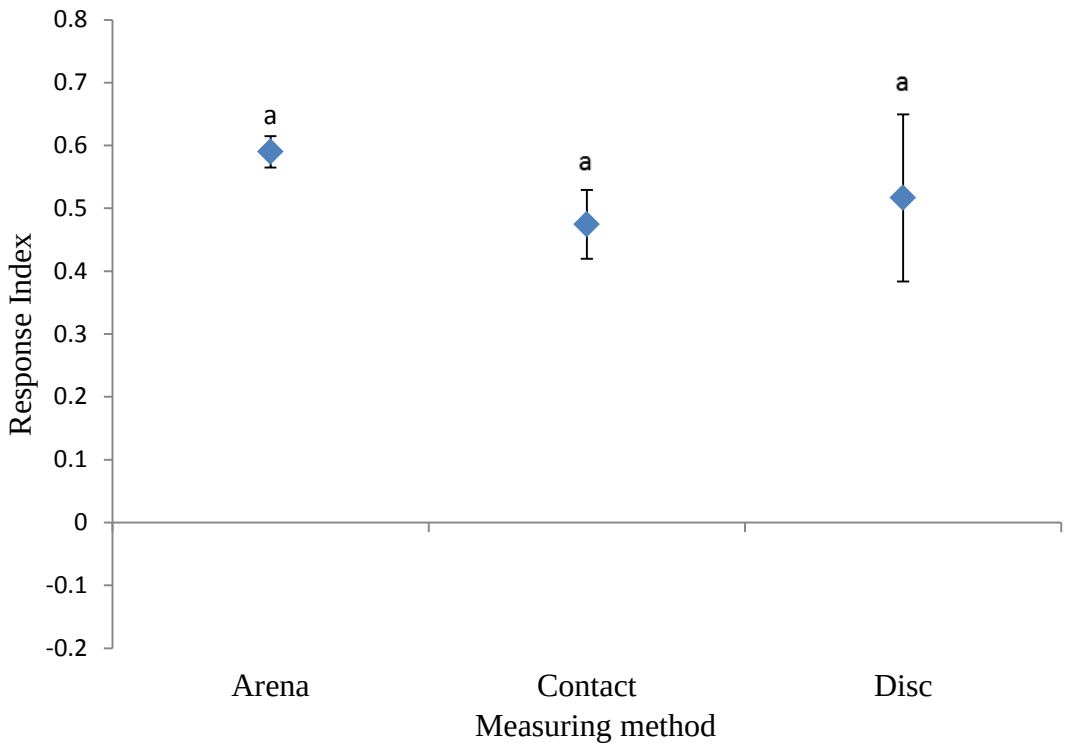

Figure 3.7 Filter disc choice bioassay. Mean response index (± SE) of adult female thrips tested against male-exposed discs using different measuring methods. Response index was calculated as (T - C)/ (T + C), where T is the number of thrips in the treatment half (exposed-disc) and C is the number of thrips present in the control half (blank disc). The whole arena (Petri dish) was divided into two equal halves of treatment and control. *Arena method*: counts of thrips found in the area within the divided halves of the petri dish. *Contact method*: number of times the test thrips touched the disc in each half. *Disc method*: counts of thrips present on the disc in the halves. Means with the same letter are not significantly different from each other (Tukey's test).

3.3.4 Thrips-exposed filter paper discs

The response index for the treatment combinations are shown in Figure 3.8. There was a significant difference between the sources (exposed discs) with both sexes responding to the male-exposed discs while female-exposed discs did not elicit a

corresponding response (ANOVA, $F_{1,47} = 479.31$, $P < 0.0001$). For the effect of sex, adult female test thrips responded more significantly than adult male thrips when exposed to the filter paper discs (ANOVA, $F_{1,47} = 5.01$, $P = 0.03$).

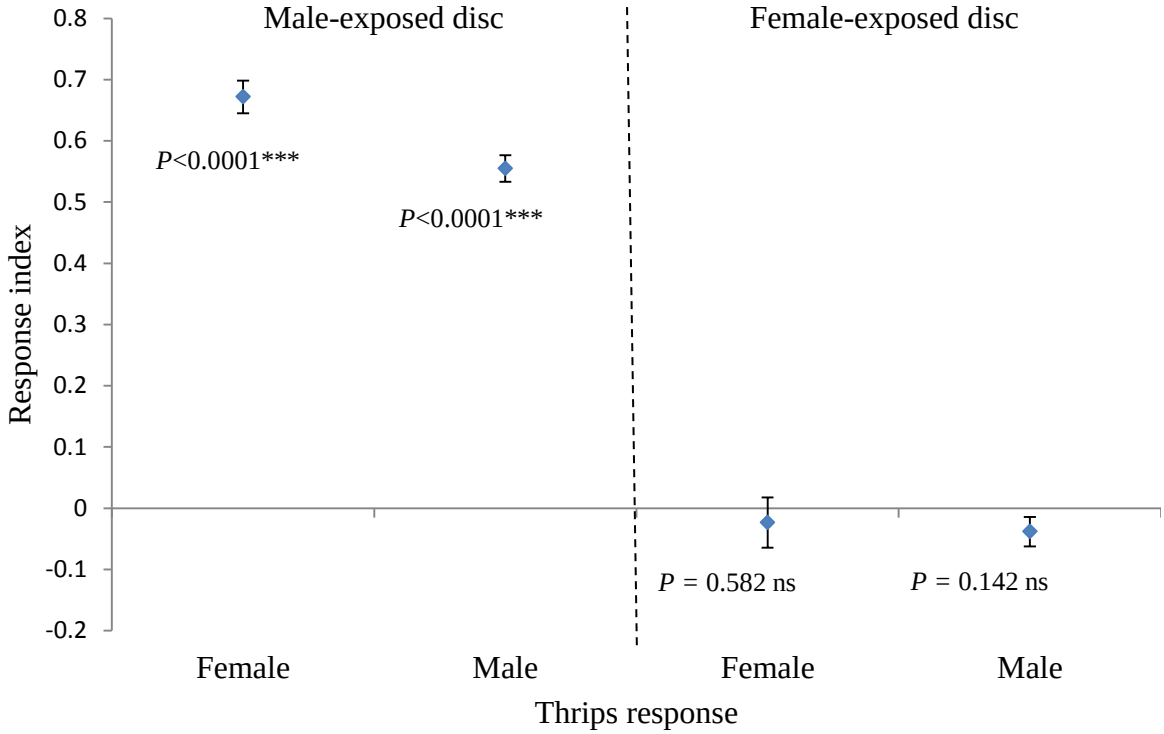

Figure 3.8 Filter disc choice bioassay. Mean response index (± SE) of different treatment combinations of adult *F. occidentalis* to exposed discs. The response index was calculated as (T - C)/ (T + C), where T is the number of test thrips in the treatment half (exposed-disc) and C is the number of test thrips present in the control half (blank disc). The petri dish was divided into two equal halves of treatment and control. Filter paper discs were exposed to mixed-age adult male and female *F. occidentalis* for a duration of 300 min. Both sexes were then tested against each exposed disc to obtain a response index. n=12 trials.

There was no significant difference from zero in the response index for male or female to female-exposed discs ($t_{(11)} = 1.58$, $P = 0.142$; $t_{(11)} = 0.57$, $P = 0.58$). However, there was a significant difference in the response index, when female *F. occidentalis* were added to

the dish containing the male-exposed disc (treatment) compared to the no-thrips exposed disc (control) ($t_{(11)} = 25.15$, $P < 0.0001$). A similar response was observed when male *F. occidentalis* were introduced ($t_{(11)} = 25.59$, $P < 0.0001$). This showed that males and females responded to male-exposed discs but not to female-exposed discs.

The track of one individual female in a choice bioassay is shown in figure 3.9.

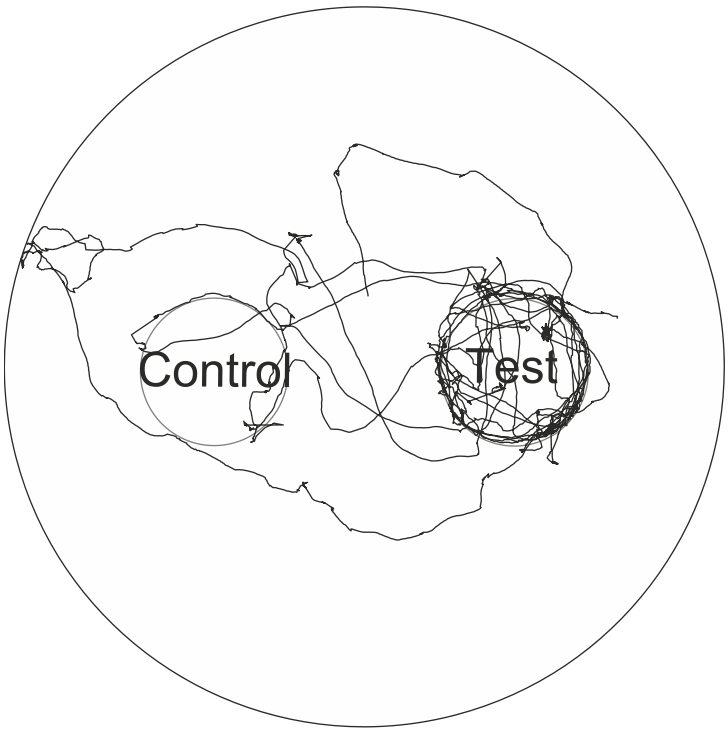

Figure 3.9 The track in the filter paper disc choice bioassay of one individual adult female *F. occidentalis* over 10 min when presented with a male-exposed disc (Test) and a non-exposed disc (Control). Occasional gaps between points are caused by short flights. The video of the track was digitized by I. A. N. Dublon.

3.3.5 Time of exposure

The time of exposure of the discs had a significant effect on the pheromone. The first set (½, 1½ and 2½ h) was significantly different (ANOVA, $F_{2,17} = 13.08$, $P = 0.012$)

and the second set (2½, 5 and 7½ h) was also significantly different (ANOVA, $F_{2,29} = 4.50$, $P = 0.026$).

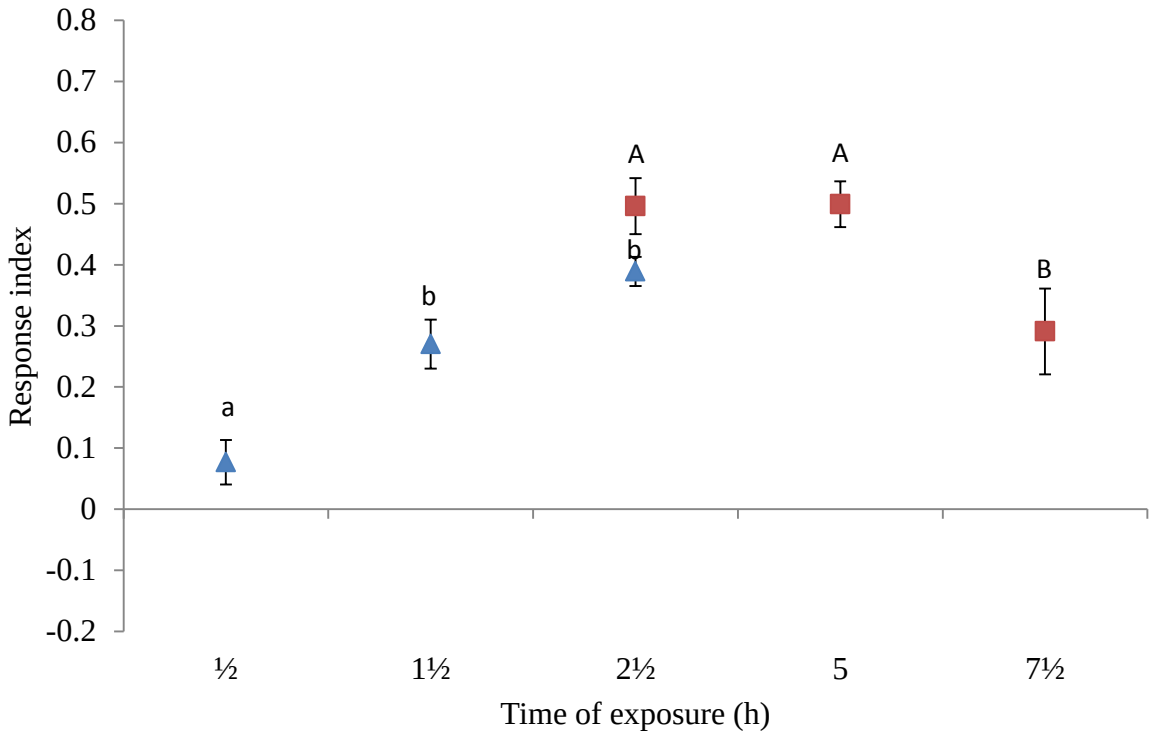

Figure 3.10 Filter disc choice bioassay. Mean response index (± SE) of mixed-age adult female *F. occidentalis* to male-exposed discs. The filter paper discs were exposed to mixed-age adult males for different times. The response index was calculated as (T - C)/ (T + C), where T is the number of test thrips in the treatment half (exposed-disc) and C is the number of test thrips present in the control half (blank disc). Two experiments were conducted (▲=29 Nov. 2010 – 13 Dec. 2010, ■ =03 Nov. 2010 – 20 Nov. 2010); within each experiment, means with the same letter are not significantly different (Tukey's test). n=12 trials.

However, the response of adult female thrips to male-exposed disc at ½ h was not significantly different from zero ($t_{(9)} = 2.12$, $P = 0.063$) but others were significantly different from zero, 1½ h ($t_{(9)} = 6.74$, $P < 0.0001$) and 2½ h ($t_{(9)} = 16.39$, $P < 0.0001$). The second set of the experiment was significantly different from zero across the three

exposure times used, 2½ h ($t_{(9)} = 10.82$, $P < 0.0001$), 5 h ($t_{(9)} = 13.38$, $P < 0.0001$) and 7½ h ($t_{(9)} = 4.14$, $P = 0.003$) (Figure 3.10). This experiment was done with the assumption that longer duration of exposure may give a higher dose thereby eliciting a greater response index.

3.4 Discussion

3.4.1 Bioassay method: Modifications

Both sexes of walking *F. occidentalis* were previously shown to be attracted to male odour (Kirk & Hamilton, 2004) and this was found with male odour on the discs (Fig. 3.8). Male-exposed discs induced a statistically significant behavioural response, while female-exposed discs did not (Fig. 3.8), validating the earlier findings of male-only compounds.

It was observed that adult female thrips survived longer than the adult male thrips (Fig. 3.4) without moisture. However, both males and females were alive throughout the period of the experiment when moisture was provided (Fig. 3.5). This result is in agreement with the findings of Laughlin (1977) that thrips given water lived for a longer time compared to those without water. The activities of thrips may explain this observed difference in male and female *F. occidentalis*. Female thrips are bigger than male thrips which is indicative of ability to store up more water in their body. Their bigger size gives lower surface area – volume ratio and this makes them less vulnerable.

In the filter paper discs bioassays, Dublon (2009) placed the water on the Parafilm membrane; whereas in the current study the water was placed on the base of the glass tube at a distance that prevented it from moistening the filter paper discs. This ensured that treatment and control discs were equally moist. An artifact was described in section 3.1.4 associated with the previous bioassay where female thrips responded to female-exposed

discs, however, this result now supports the finding that female *F. occidentalis* do not produce any volatile pheromones.

F. occidentalis is active during the day with little or no flight activity at night (Kiers *et al.*, 2000). Male *P. kellyanus* form aggregations towards late afternoon and females are attracted for mating (Mound & Jackman, 1998). Based on these findings, exposing filter discs to males to coincide with this suggested period probably reflects the mating period where aggregations do occur. Dublon (2009) exposed filter discs to male thrips for 24 ± 2hours, while in this experiment the filter discs were exposed for 5 hours by day only. The new improved bioassay not only showed significant results but also allowed high productivity as many experiments could be carried out within the time period.

The three measurement methods all confirmed that thrips responded to compounds on the filter paper disc. Though, similar RI was obtained for the methods, they may be detecting different types of response; this possibility needs to be examined in more detail. The differences observed suggest the method may be measuring different response to the compounds. The arena method suggests an attraction towards the odour source; presumably the method can detect a long range compound. “Contact” and disc methods seem to show arrestment when they are in contact with the exposed disc. The time spent on or beneath the disc suggests the presence of short-range compounds which can only be detected until there is a contact (touching). This needs further studies to unravel this phenomenon. The arena method is sensitive, practical and clear in measuring response. With the arena method, thrips were able to respond and choose a preferred side quickly and the responses were clear enough to see which side they preferred without any ambiguity. It measured the preference of test thrips to an area with a volatile odour which is similar to in the field. The insect does not need to have contact with an odour before responding to it, most especially long range compounds. There is a large effect of response with low

variability (Figure 3.7) meaning that behavioural responses can be detected with a small number of replicates. It further takes into account the total population of test thrips involved in the experiment thereby making the statistical analysis robust but this cannot be said about the other two methods. However, the arena method may be difficult to use depending on the nature and size of the arena, because saturation of the arena with odour may present a false result where test thrips may be accepting the control side due to the mix of the odour. It is therefore important to measure over a range of concentrations to remove bias from odour mixing. At high concentration, the odour will spread across the whole dish and no treatment effect will be detected

The idea of measuring contact may be misleading, but it could be a response to a localised compound instead of a long-range compound. For example, it was possible for a single thrips to be in contact with the filter discs throughout the span of the experiment, whereas other thrips may be involved in walking around the dish. This means the measurement may be from just one thrips out of the entire group of thrips. The contact may be a result of hesitation, not making a decision which does not reflect a stronger response. Multiple records of an individual are very high using the contact method. Unequal weight of discs may be a problem when using the disc method, thrips may respond to the size rather than the compound on the disc. The pheromone production and response in the field does not suggest that the disc method is appropriate, because one of the compounds, neryl (*S*)-2-methylbutanoate is a long-range compound which attracts from a distance. However, the response observed with the disc method suggested that thrips were possibly responding to another compound and this calls for further investigation to detect if there are other possible compounds on the male-exposed discs. All the methods are indicative of a response though more replicates may be needed for contact and disc methods to reduce the variability observed. However, based on the track obtained (figure 3.9) with the exposed-

discs, there is a strong indication that one of the male-produced compounds may be responsible for the increased turning frequency or angle when walking thereby resulting in convergence on the source of the compound.

The statistical analysis method used eliminates the problem associated with the response index as mentioned by Hare (1998), Kroon and Housefield (2003), James et al., (2008) and Takakura (2009). The Response index solved the problem of multiple records of an individual by ensuring all thrips were used in the analysis. The index obtained was treated as a replicate to eliminate pseudoreplication. This satisfies the underlying assumptions of independent data and normality.

Figure 3.8 shows that male *F. occidentalis* produced a compound or compounds that were not produced by female *F. occidentalis*. However, there was no response of both male and female *F. occidentalis* to female-exposed discs (Fig. 3.8) suggesting that females do not produce any known compounds, which disagrees with the finding of Dublon (2009) for female *F. occidentalis* attraction.

From the bioassay it may be assumed that male-produced compounds are contact, air borne compounds or a combination. The "contact" and "disc" methods suggest the possibility of a non-volatile compound or a short-range compound on the filter paper disc because test thrips responded to the exposed disc. The possibility of an additional compound to neryl (*S*)-2-methylbutanoate and (*R*)-lavandulyl acetate on the male-exposed discs is fully discussed in chapters 4 and 5.

3.4.2 Time of exposure

The time of exposure reveals that adult female *F. occidentalis* can respond to the male-produced compounds present on the filter paper over a range of doses (Figure 3.9). The response to the male-exposed disc at ½ h was not statistically significant. The response

index was increasing as the time of exposure increased, however, at 7½ h, there was a reduction in the effect which could be due to the over saturation of the arena used for the experiment. The adult female *F. occidentalis* would have been unable to distinguish between the two halves of treated and control side, thereby responding to both sides of the Petri dish arena. However, increasing the size of the bioassay arena would remove this effect and could be used to test whether saturation is the cause of the decrease in response at 7½ h.

The two sets of the experiment were done at different dates; comparisons between the two sets may not be appropriate because responses fluctuate. The disc exposed for 2½ h, which is common to both sets, shows no significant difference but this may not be entirely true because thrips behaviour has been shown to be very much affected by day to day variation (O'Leary, 2005). This has shown that thrips can detect and respond accordingly to male-exposed discs; however, it would be of interest to determine how concentration affects thrips response. Different doses of synthetic compounds were used to determine this dose response (see Chapter 4).

3.5 References

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Chapter 4

Behavioural responses to natural and synthetic pheromones on an artificial substrate

4.1 Introduction

The development of novel methods of insect control or the improvement of the existing methods necessitates the careful study of insect behaviour. Such behaviour can be exploited to reduce damage to the crop and the environment. The ban on many chemical pesticides promotes the study of alternative ways to combat the damage caused by insects. Semiochemicals are a promising method for monitoring and control of insect pests. Therefore, it is necessary to study the effects of semiochemicals on insect behaviour both in the laboratory and in the field with a view to understand the interactions involved. This can be done in the laboratory using bioassays and in the field through the use of traps. Though it is believed that laboratory bioassays cannot fully explain the interactions in the field, they can provide basic indications needed to understand such behaviour. It is therefore important to carry out both laboratory and field work to make valid conclusions.

Two major volatile compounds have been identified in male *F. occidentalis*, neryl (*S*)-2-methylbutanoate, an aggregation pheromone known to attract both females and males in the field and (*R*)-lavandulyl acetate which reduced thrips catches in the field (Hamilton *et al.*, 2005). While neryl (*S*)-2-methylbutanoate is known for its attraction, the role of (*R*)-

lavandulyl acetate still remains unknown. Based on this, it is important to further examine the behavioural response to these compounds in the laboratory with a view to understanding their specific role. This could increase the effectiveness of traps in the field.

Displacement of insects in space has been used to describe odour orientation. Two forms of orientation have been described, taxes and kineses to understand the behavioural response of insects to an odour source (Shorey, 1973). Tactic orientation describes the path of an insect directed towards, away from or at a fixed angle to an odour source while kinetic orientation involves the changes of locomotion rate, turning rate or time spent on locomotion (Shorey, 1973). Using the stimulus involved, chemotaxis occurs when an insect responds to an odour source while photaxis, geotaxis and anemotaxis indicate responses to light, gravity and air currents respectively. Also, orthokinesis occurs where an insect does not use the direction of odour gradient but is actively stimulated to move at different rates while klinokinesis involves turning at different frequencies. Simple classification of orientation movement may not be entirely correct because insects may use integrated mechanisms at the same time or swiftly change these mechanisms at a different time (Rust & Bell, 1976; Hare, 1998). To separate these orientation movements, behaviourally discriminating bioassays have been extensively used to separate various components of orientation approach by the insects.

Many studies have used walking and flight bioassays to understand the behavioural responses of thrips to semiochemicals (Webster *et al.*, 2006; Koschier *et al.*, 2007; Dublon, 2009; Davidson *et al.*, 2012). The natural activity of insects in the field should determine the kind of bioassays to be used for screening of semiochemicals in the laboratory. High flying insects may be poorly bioassayed if a still air (see section 3.1.2) method is used to study the flight activity in the laboratory. Responses of *F. occidentalis* have been reported extensively using these bioassay methods (Koschier *et al.*, 2000, 2007; Webster *et al.*,

2006; Dublon, 2009; Lim & Mainali, 2011; Davidson *et al.*, 2012) through the use of Y-tube olfactometer, petri dish arena, glass tube and flight chambers in the laboratory to understand their behaviour to different cues (visual, olfactory and host). Although, *F. occidentalis* is a weak flying insect, the flight response has been measured using take-off or short flights (flits). Walking or flying responses have been measured as the number of thrips that move (walk/fly) towards the source of the odour.

4.1.1 Experimental aims

This chapter attempts to examine the behavioural responses of adult female *F. occidentalis* in a laboratory condition using the improved bioassay (see Chapter 3). This is to understand their preference in a choice bioassay which can then be used to determine the role of these compounds. The no-choice bioassay was used to measure the walking and flit responses of female *F. occidentalis*. This was done to understand the specific role of the compounds whether they increase or decrease the activity level of female *F. occidentalis*. Both natural (male-exposed discs) and synthetic compounds were assayed. This chapter also attempts to reproduce the behavioural response to natural pheromone. By using synthetic compounds, this could indicate the compounds present. However, if this is not possible, detection of other possible compounds will then be carried out using GC-MS (chapter 5).

4.2 Materials and methods

Both natural (male-exposed discs) and synthetic compounds of those produced by adult male *F. occidentalis* were assayed in the same way as with the filter paper disc choice bioassay used in the development of pheromone bioassay in chapter 3. A wide range of doses of the synthetic compounds, neryl (*S*)-2-methylbutanoate and (*R*)-lavandulyl acetate were applied on filter paper discs to examine their effects on the female

F. occidentalis to determine whether they were an attractant or not. This bioassay attempts to detect any biological response of female *F. occidentalis* to pheromone. To gain more understanding of their response to these compounds, a no-choice bioassay was further used to measure activity level to elucidate the specific function of these compounds thereby suggesting possible ways of utilization in the field.

4.2.1 Choice bioassay of natural pheromone: obtaining pheromone

To test the behavioural response to natural pheromone on an artificial substrate, filter paper discs were exposed to 15 male *F. occidentalis* for 300 minutes as described in section 3.2.2. The exposure was carried out in the early hours of the day from 08:30 am – 2:30 pm to follow the period already established that thrips produce pheromone in the laboratory (see Chapter 3).

The filter paper disc-choice bioassay (see 3.2.3) was used. Male-exposed and control cellulose filter discs were placed on the two positions marked on the Petri dish using forceps cleaned with hexane (*n*-hexane, pesticide residue analysis grade (1526764), VWR International Limited, Poole, UK) as described in 3.2.3.

4.2.2 No-choice bioassay of natural pheromone: obtaining pheromone

To detect the behavioural response (walking and flits) of female *F. occidentalis* both in the presence and absence of pheromone, a no-choice test was used. Filter paper discs were exposed to 15 male *F. occidentalis* to obtain male-exposed discs as described in 4.2.1. Thereafter, a set of four 40 mm Petri dishes and corresponding lids (Anumbra, Fisher Scientic, UK) were prepared as described in 2.4. There were two sets of experiment done consisting of treatment and control dish each. Required numbers (3 mixed-age adult female per bioassay) of *F. occidentalis* were aspirated and anaesthetised by exposing them to a gentle 10 s stream of carbon dioxide (British Oxygen Company, UK) and transferred

to the middle of the Petri dishes. Three mixed-age females, rather than 15, were used per bioassay to make observation and monitoring easier. The lids were added and Parafilm membrane was stretched round the edges of the Petri dishes covering both the base and the lid in order to create a tight seal against thrips escape. Observation and recording were done for 30 minute period as described in 3.2.1. The bioassay room was maintained at a constant temperature of 25°C ± 2°C as described in 3.2.2.

4.2.3 Choice bioassay of synthetic pheromone: preparation and application

The filter paper disc-choice bioassay (see 3.2.3) was used to study the response of female *F. occidentalis* to synthetic compounds. Two cellulose filter discs (Whatman International Limited (1001020) grade 1, 20mm diam.) were placed on the two equidistant positions marked on the Petri dish as described in 3.2.3.

Fifteen mixed-age adult female (per bioassay) of *F. occidentalis* were aspirated and anaesthetised by exposing them to a gentle 10 s stream of carbon dioxide (British Oxygen Company, UK) and transferred to the middle of the Petri dish containing the treatment and control. The treatment disc was injected with a 5 µl of synthetic compound in hexane and a 5 µl of hexane was added to the corresponding control. This application was done in a fume cupboard in the Chemical Ecology laboratory. The compounds were applied as a single drop to the middle of the filter paper discs and allowed to spread to the edge before taken to the bioassay room.

The lids were added and Parafilm membrane was stretched round the edges of the Petri dish as described in 3.2.3. Treatment and control filter disc positions on the Petri dishes were randomly assigned using random numbers within each replicate to reduce any possible directional room bias. This was carried out in a bioassay room with a constant temperature of 25°C ± 2°C as described in 3.2.2.

A range of doses of synthetic compounds, neryl (*S*)-2-methylbutanoate and (*R*)-lavandulyl acetate, was used to detect if any responses were present when female *F. occidentalis* is introduced into the Petri dish arena containing the filter paper discs. The doses used were 50 pg (5 µl of 10 pg μl^{-1}), 500 pg (5 µl of 100 pg μl^{-1}), 5 ng (5 µl of 1 ng μl^{-1}), 50 ng (5 µl of 10 ng μl^{-1}) and 500 ng (5 µl of 100 ng μl^{-1}).

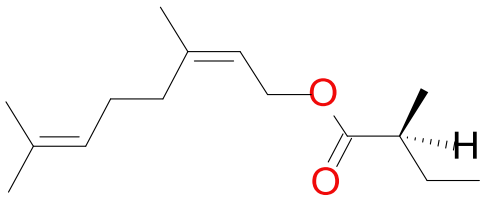

Figure 4.1: Chemical structure of Neryl (*S*)-2-methylbutanoate

4.2.4 No-choice bioassay of synthetic pheromone: preparation and application

A set of four 40 mm Petri dishes and corresponding lids (Anumbra, Fisher Scientic, UK) were prepared as described in 2.4. Required numbers (3 mixed-age adult female per bioassay) of *F. occidentalis* were aspirated and anaesthetised as described previously in 3.2.2. The treatment disc was injected with a 5 µl of synthetic compound in hexane and a 5 µl of hexane was added to the corresponding control. The lids were added and Parafilm membrane was stretched round the edges of the Petri dish as described in 4.2.2. Treatment and control filter disc positions on the Petri dishes were randomly assigned using random numbers within each replicate to reduce any possible directional room bias. This bioassay was carried out in the designated room as described in 4.2.1. Range of doses used was the same as mentioned in 4.2.3.

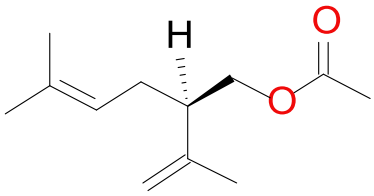

Figure 4.2: Chemical structure of (*R*)-lavandulyl acetate

4.2.5 No-choice bioassay of synthetic pheromone: response of adult female *F. occidentalis* to (*R*)-lavandulyl acetate and (*S*)-lavandulyl acetate

This was done in a similar manner as described in 4.2.2 with the exception that only one dose (500 pg) was used to compare the behavioural response of adult female *F. occidentalis* when presented with (*R*)-lavandulyl acetate or (*S*)-lavandulyl acetate. This dose was chosen because it gives a significant response with (*R*)-lavandulyl acetate when tested alone.

4.2.6 No-choice bioassay of synthetic pheromone: response of adult male *F. occidentalis* to (*R*)-lavandulyl acetate.

This bioassay was carried out as described in 4.2.2; this was done to compare the behavioural response of adult male and adult female *F. occidentalis* to different doses of (*R*)-lavandulyl acetate.

4.2.7 Observations

As described in 3.2.1, the arena measurement method was used throughout the choice bioassays. The Petri dish was divided into two equal halves using the treatment and control filter discs as the basis of division. The number of adult female thrips found on each half of the Petri dish was measured visually and recorded. Records were taken every 3 min for a total of 30 min giving 10 recordings per bioassay. However, other behavioural activities were observed and noted for any variations which may assist in the interpretation of the results.

For no-choice bioassays, the number of thrips walking within the whole arena was recorded as a walking response while attempted flight and landing was measured as a flit. These were recorded over a 30 min period. A walking response was recorded when a thrips moves within the arena over glass surface or filter paper disc. Flits were recorded as an event when a thrips leaves any part of the arena with an attempted flight/take-off and landing.

4.2.8 Statistical analysis

Choice bioassays: The number of adult female thrips found on each half of the Petri dish was measured visually and recorded. Data collected were averaged for each bioassay and processed to obtain a Response index (RI) as described in 3.2.5. The RI was analysed using a one sample *t*-test for natural pheromone and General Linear Model ANOVA (GLM) for concentrations of synthetic pheromone. All data were tested for normality as described in 2.7.

No-choice bioassays: The walking and flit responses of female *F. occidentalis* were recorded as described in 4.2.7. The walking response data was analysed using General Linear Model ANOVA and Tukey's test was used to compare walking response across the treatments. Flits data were not normally distributed due to the presence of extreme values. Log transformation was then applied, which normalized the data, before using General Linear Model ANOVA and Tukey's test was used to compare treatments against control.

4.3 Results

4.3.1 Response of adult female *F. occidentalis* to natural pheromone in a choice bioassay

As explained in Chapter 3, the response index was used to measure the behavioural response. The calculated response index of adult female *F. occidentalis* to male-exposed discs and non-exposed disc showed a significant difference from zero (section 3.3.4). This

reveals that adult female *F. occidentalis* responded positively to male-exposed discs. This preference for male-exposed discs indicates the presence of some active compounds which they were able to detect.

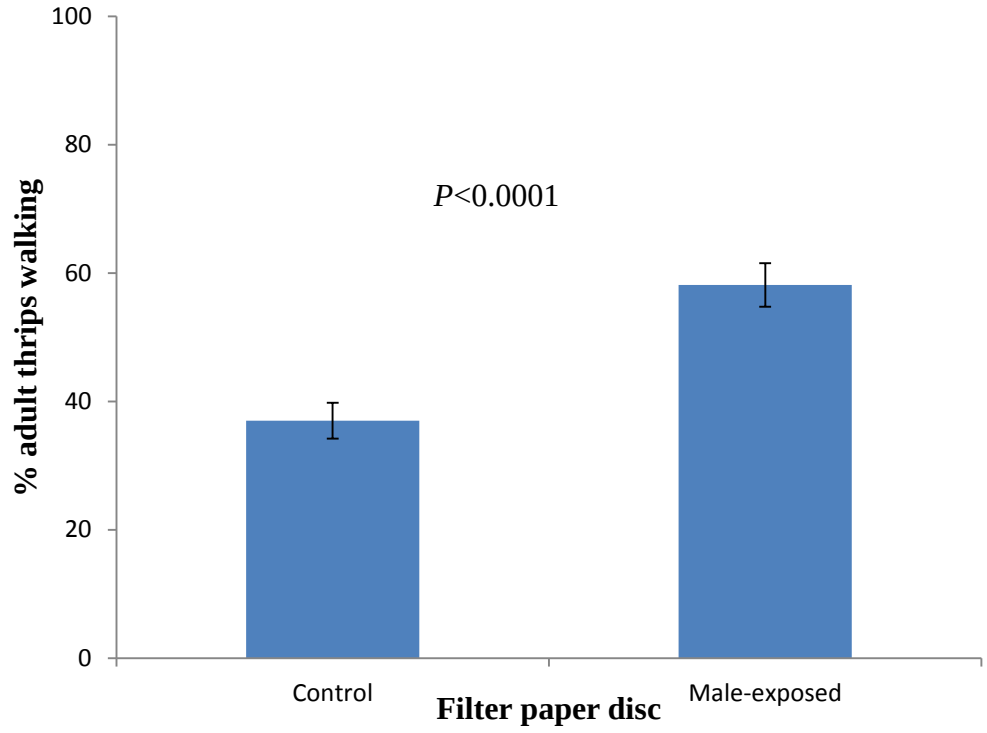

Figure 4.3 The mean (± SE) percentage of adult female *F. occidentalis* walking on the filter disc during the no-choice bioassay period of 30 min. There was significantly more walking activity of adult female *F. occidentalis* on the male-exposed disc than on the control disc (ANOVA, $F_{1,29} = 23.48$, $P < 0.0001$).

4.3.2 Response of adult female *F. occidentalis* to natural pheromone in a no-choice bioassay

Adult female *F. occidentalis* were observed when presented to male-exposed and non-exposed discs. The observations recorded were walking and flit responses in the presence of a disc with (male-exposed) and without (no thrips-exposed) pheromone. The walking response of adult female *F. occidentalis* was significantly higher with male-exposed discs (ANOVA, $F_{1,29} = 23.48$, $P < 0.0001$) (Figure 4.3). A similar result was

recorded for the flit response. There was significantly more flitting by adult female *F. occidentalis* presented with a male-exposed disc than a no-thrips exposed disc (ANOVA, $F_{1,29} = 7.52$, $P = 0.011$) (Figure 4.4). These results showed that adult female *F. occidentalis* can detect active compounds on the male-exposed disc and respond to it by an increase in walking and flitting.

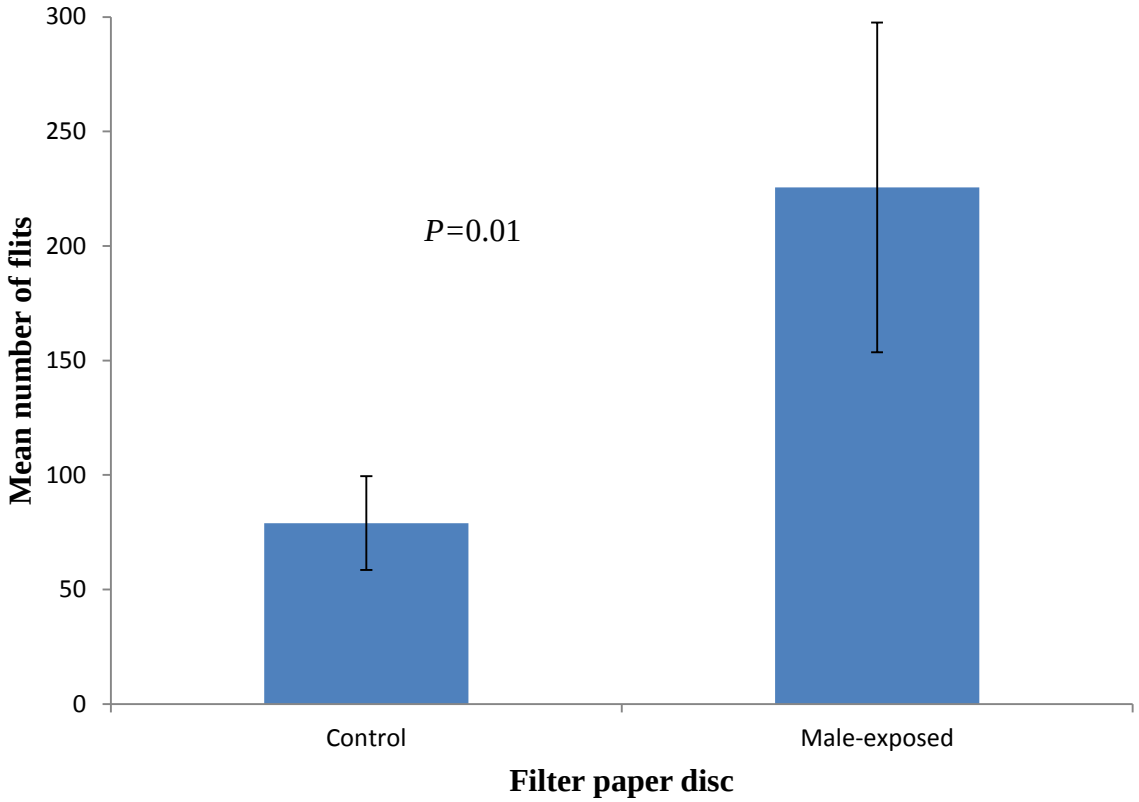

Figure 4.4 The mean (± SE) number of flits by adult female *F. occidentalis* on the filter disc during the no-choice bioassay period of 30 min. There was significantly more flit activity of adult female *F. occidentalis* on the male-exposed disc than on the control disc (ANOVA, $F_{1,29} = 7.52$, P=0.01).

4.3.3 Response of adult female *F. occidentalis* to synthetic pheromone in a choice bioassay

To further evaluate the response of adult female *F. occidentalis*, the synthetic form of the identified compounds from adult male *F. occidentalis* was used. This was done to determine if the effect of the natural pheromone found on the male-exposed disc can be

reproduced with synthetics. To capture the overall effect, five different doses were used throughout the experiment. And as discussed earlier, the response index was used to measure their level of response (see Chapter 3).

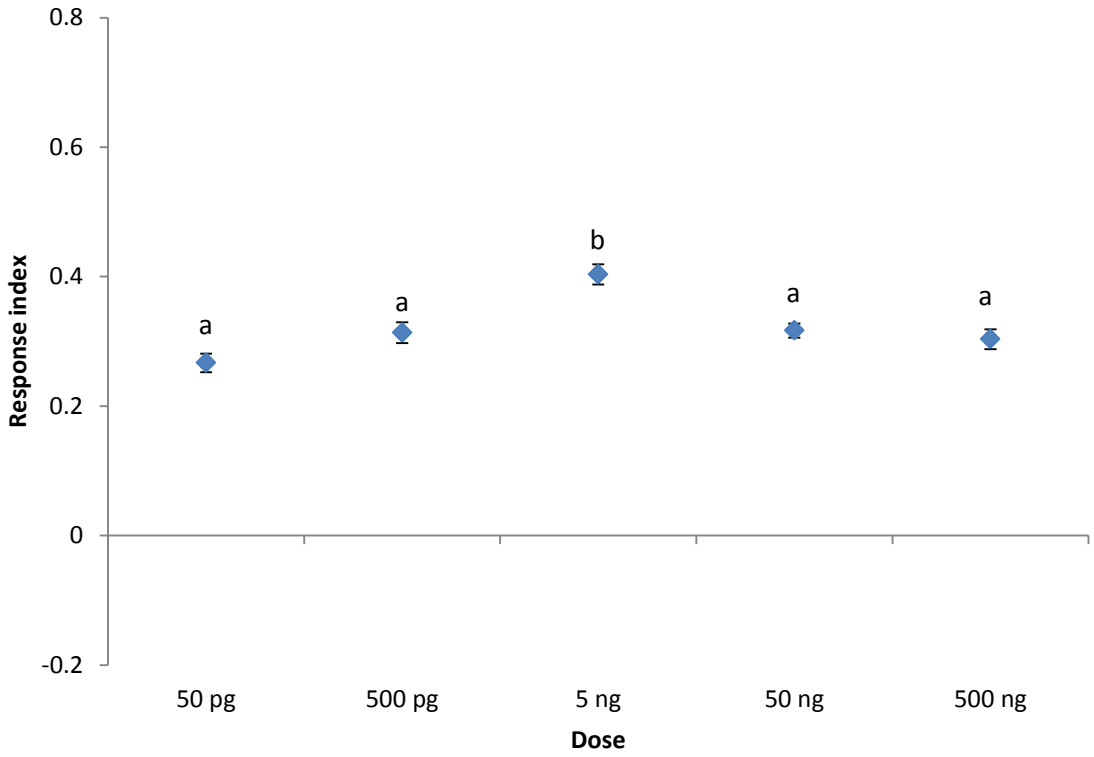

Figure 4.5 Filter paper disc choice bioassay. Mean response index (± SE) of mixed-age adult female *F. occidentalis* to discs treated with different concentration of neryl (*S*)-2-methylbutanoate. Response index was calculated as (T - C)/ (T + C), where T is the number of test thrips in treatment half (treated-disc) and C is the number of test thrips present in the control half (blank disc). Means with the same letter are not significantly different at P<0.05 (Tukey), n= 12 trials. The mean RI at all doses was significantly different from zero (*P*<0.0001 in all cases).

Adult female *F. occidentalis* was given the chance to choose between the filter paper disc injected with synthetic pheromone and the one without pheromone which served as the control disc. For neryl (*S*)-2-methylbutanoate, adult female *F. occidentalis* responded positively to the filter paper disc with pheromone thereby showing a significant response

(ANOVA, $F_{4,59}$ = 11.88, P <0.0001) (Figure 4.5). However, 5 ng showed the highest response index (0.4) among the 5 doses while the lowest (0.27) was given by 50 pg.

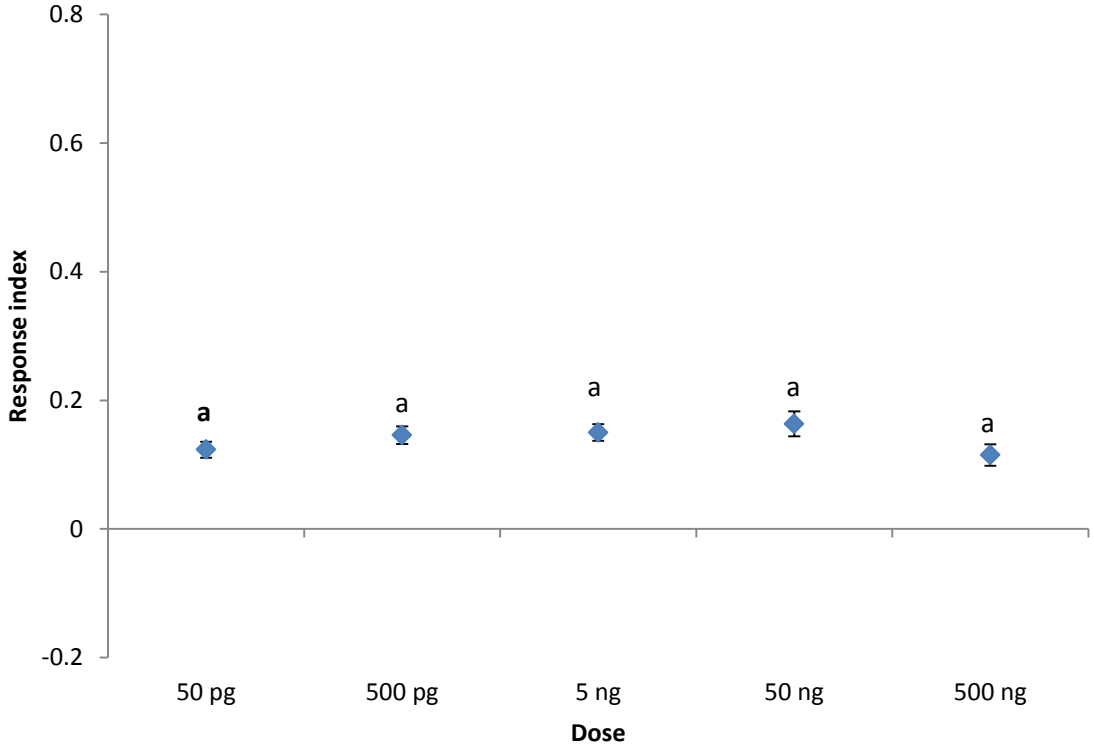

Figure 4.6 Filter paper disc choice bioassay. Mean response index (± SE) of mixed-age adult female *F. occidentalis* to discs treated with different concentration of (*R*)-lavandulyl acetate. Response index was calculated as (T - C)/ (T + C), where T is the number of test thrips in treatment half (treated-disc) and C is the number of test thrips present in the control half (blank disc). Means with the same letter are not significantly different at P<0.05 (Tukey), n= 12 trials. The mean RI at all doses was significantly different from zero (*P*<0.001 in all cases).

When (*R*)-lavandulyl acetate was presented to adult female *F. occidentalis*; a response was elicited by treated discs significantly more than control discs (Figure 4.6). All the doses were significantly different from zero (Figure 4.6). This clearly showed that (*R*)-lavandulyl acetate can be detected from a short distance. However, there was no significant difference

between the treatments (ANOVA, $F_{4,59}$ = 1.71, P = 0.16). Adult female *F. occidentalis* significantly choose the pheromone side more than the side without pheromone across all the tested concentrations. The highest response index of 0.16 was recorded with 50 ng while the lowest 0.11 was given by 500 ng (Figure 4.6).

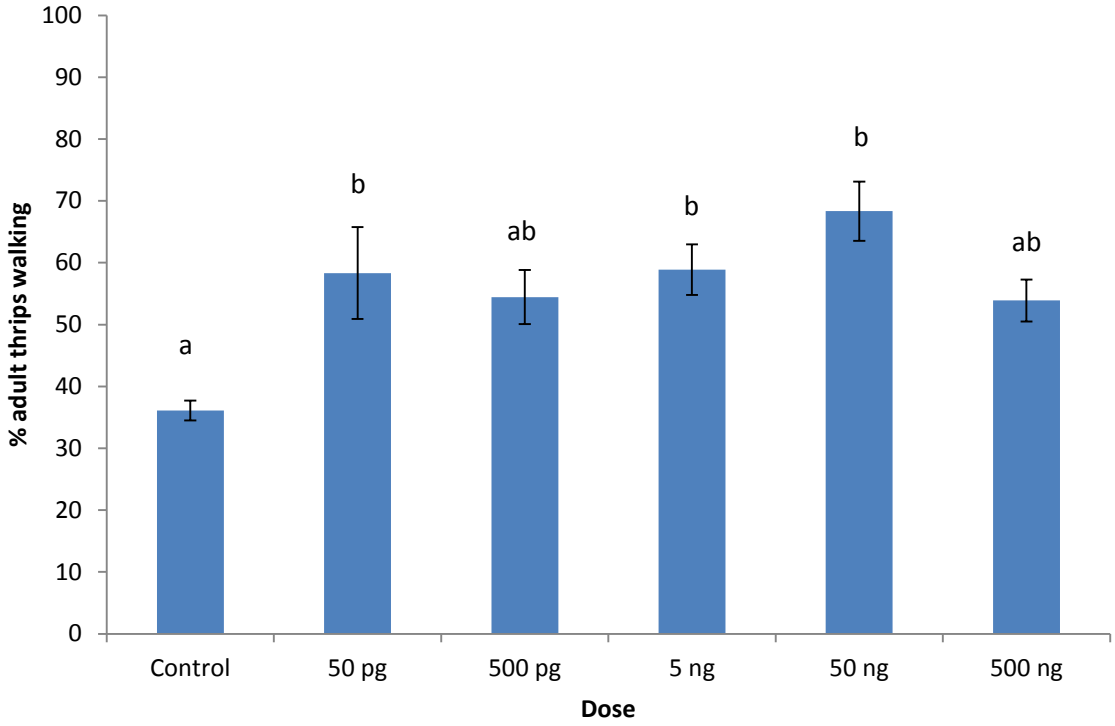

Figure 4.7 The mean (± SE) percentage of adult female *F. occidentalis* walking on the filter paper disc during the no-choice bioassay period of 30 min. The filter paper discs were injected with neryl (*S*)-2-methylbutanoate. There was a significant difference between the walking activity of adult female *F. occidentalis* on the treated discs and the control disc (ANOVA, $F_{5,35}$ = 5.06, P= 0.002). n=12 trials.

4.3.4 Response of adult female *F. occidentalis* to synthetic pheromone in a no-choice bioassay

As discussed in 4.3.2, walking and flit responses were used to measure the behavioural response of adult female *F. occidentalis* to synthetic pheromone applied on a filter paper disc. Five different doses were used in this bioassay as discussed earlier. When

neryl (*S*)-2-methylbutanoate was tested, the mean number of adult female thrips walking was significantly different across the treatments (ANOVA, $F_{5,35}$ =5.06, P=0.002). As presented in figure 4.7, the highest mean number of thrips walking was recorded at 50 ng while the lowest was recorded with the control disc.

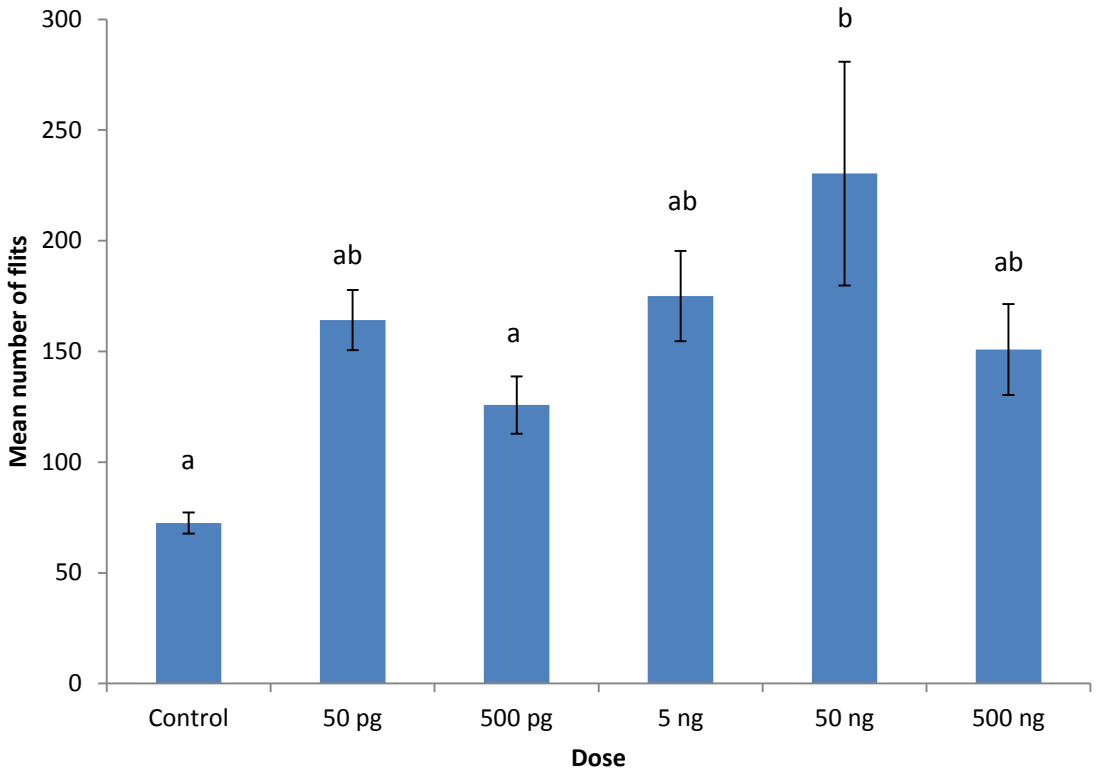

Figure 4.8 The mean (± SE) number of flits by adult female *F. occidentalis* on the filter disc during the no-choice bioassay period of 30 min. The filter paper discs were injected with neryl (*S*)-2-methylbutanoate. There was a significant difference between the flit activity of adult female *F. occidentalis* on the treated discs and the control disc (ANOVA, $F_{5,35}$ = 4.98, P= 0.003). n=12 trials.

The mean number of thrips walking on the 50 ng disc was about twice the number recorded on the control showing that the female thrips were more active in the presence of the compound. But 50 pg, 5 ng and 50 ng were significantly different from the number recorded on the control disc. As shown in figure 4.8, the flit response of adult female *F.*

occidentalis to neryl (*S*)-2-methylbutanoate was significantly different across the treatments (ANOVA, $F_{5,35}$ =4.98, P=0.003). The highest number of flits was recorded with 50 ng and is three times the number of flits recorded on the control disc which was the lowest. The highest number of flits was significantly higher than the lowest recorded flits on the control disc.

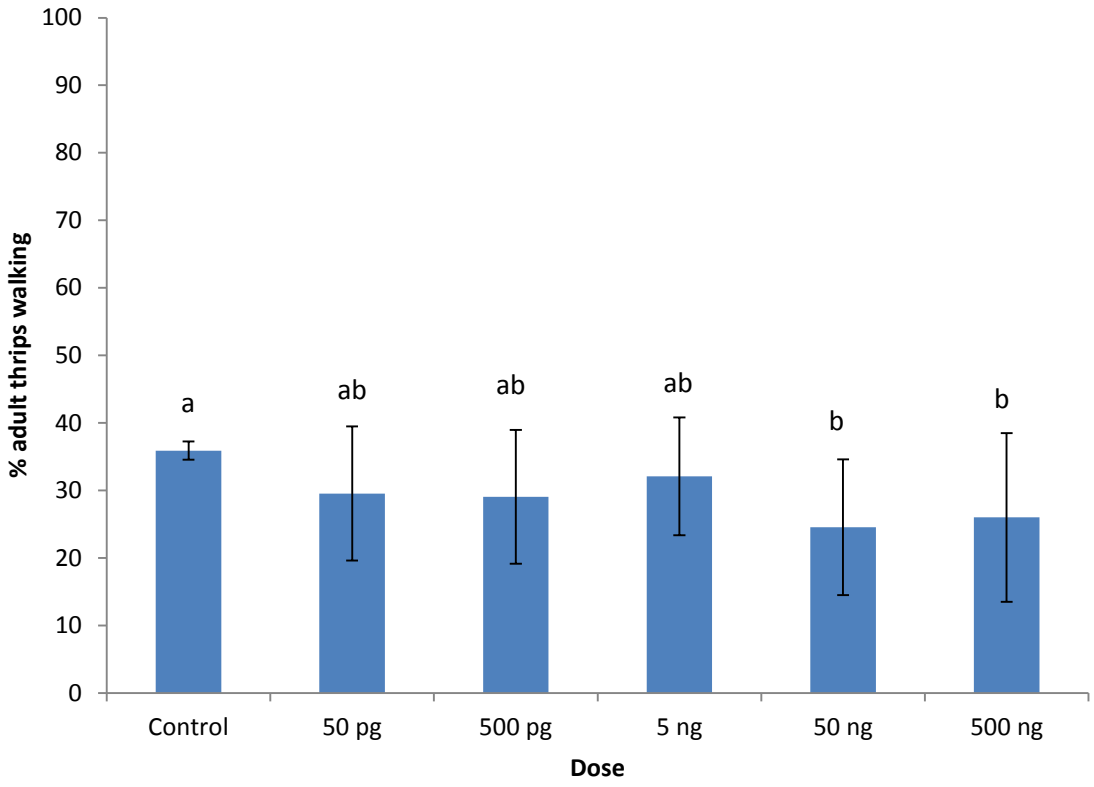

Figure 4.9 The mean (± SE) percentage of adult female *F. occidentalis* walking on the filter paper disc during the no-choice bioassay period of 30 min. The filter paper discs were injected with (*R*)-lavandulyl acetate. There was a significant difference between the walking activity of adult female *F. occidentalis* on the treated discs and the control disc (ANOVA, $F_{5,35}$ = 3.01, P= 0.029). n=12 trials.

For (*R*)-lavandulyl acetate, the mean number of adult female *F. occidentalis* walking was also significantly different across the treatments (ANOVA, $F_{5,35}$ =3.01, P=0.029). Adult female *F. occidentalis* walk less compared to the control disc, the lowest was recorded on

50 ng while the control disc gave the highest mean number of walking thrips (Figure 4.9). When adult female *F. occidentalis* was tested against (*R*)-lavandulyl acetate, the number of recorded flits was significantly less than the number on the control disc (ANOVA, $F_{5,35}$ =11.36, P<0.0001) (Figure 4.10). The highest number of flits was recorded on the control disc while the lowest was recorded on the disc with 500 ng. Number of flits recorded on all the five doses was significantly less than that recorded on the control disc.

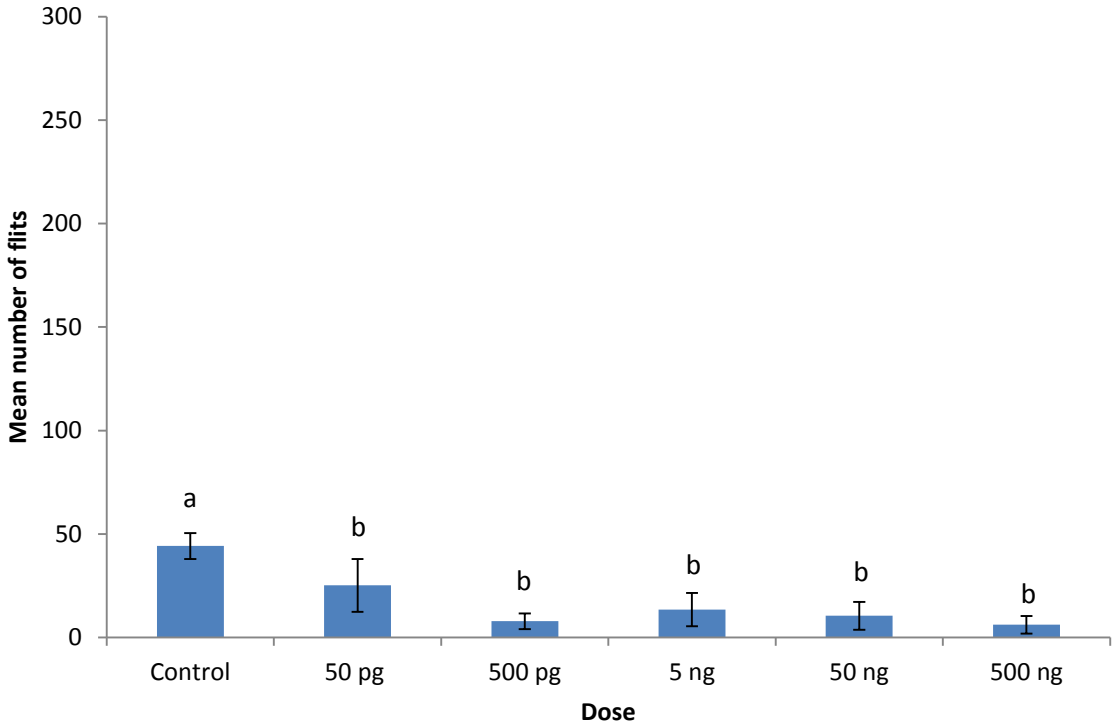

Figure 4.10 The mean (± SE) number of flits by adult female *F. occidentalis* on the filter disc during the no-choice bioassay period of 30 min. The filter paper discs were treated with (R)-lavandulyl acetate. There was a significant difference between the flit activity of adult female *F. occidentalis* on the treated discs and the control disc (ANOVA, $F_{5,35}$ = 4.98, P= 0.003). n=12 trials.

4.3.5 Response of adult female *F. occidentalis* to (*S*)-lavandulyl acetate in a no-choice bioassay

To further analyse the behavioural response of adult female *F. occidentalis,* (*R*)-lavandulyl acetate and (*S*)-lavandulyl acetate were used in this bioassay. This is to detect if

there can be any chiral differences in the two compounds. The two compounds are enantiomers of lavandulyl acetate, a chiral compound with both *R* and *S* forms. However, (*S*)-lavandulyl acetate is not being produced by thrips and was not expected to have any effect on adult female *F. occidentalis* or to be a complete opposite to what (*R*)-lavandulyl acetate would do.

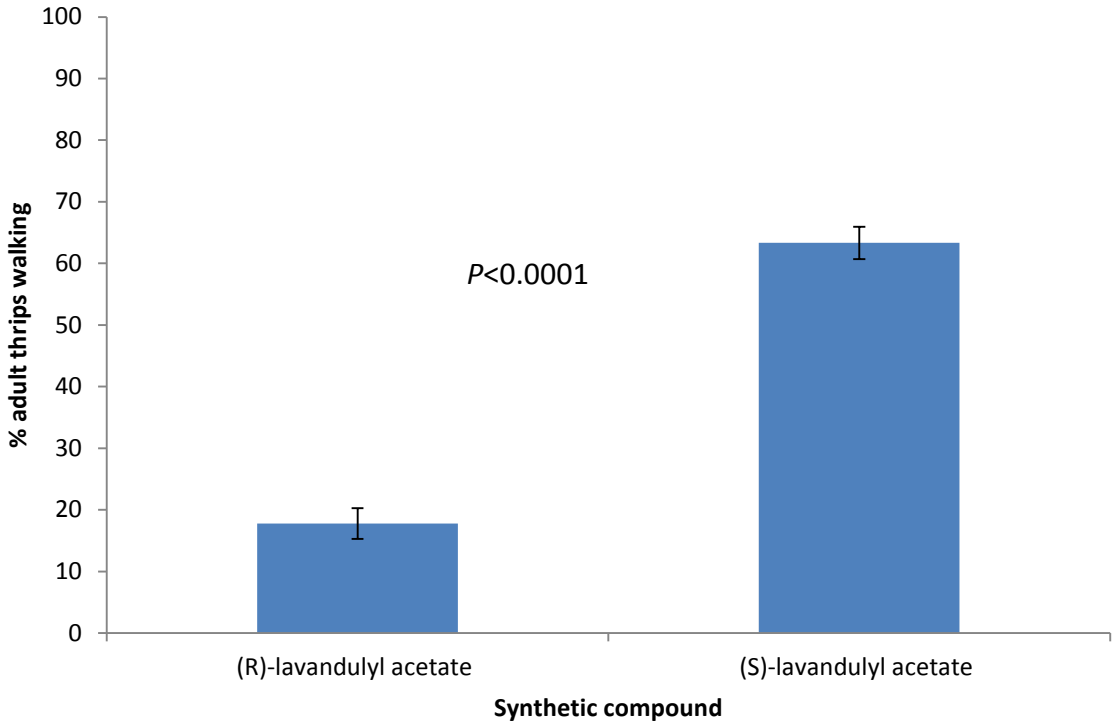

Figure 4.11 The mean (± SE) percentage of adult female *F. occidentalis* walking on the filter paper disc during the no-choice bioassay period of 30 min. The filter paper discs were injected with 500 pg each of (*R*)-lavandulyl acetate and (*S*)-lavandulyl acetate. There was a significant difference between the walking activity of adult female *F. occidentalis* on the (*R*)-lavandulyl acetate treated disc and (*S*)-lavandulyl acetate treated disc (ANOVA, $F_{1,23} = 157.4$, $P < 0.0001$). n=12 trials.

The walking response followed the same trend as flit response, more female thrips walking was recorded with (*S*)-lavandulyl acetate compared to (*R*)-lavandulyl acetate and this was significantly different (ANOVA, $F_{1,23} = 157.37$, $P < 0.0001$) (Figure 4.11).

The mean number of flits was significantly different between the two compounds (ANOVA, $F_{1,23}$ =70.54, P<0.0001). Adult female *F. occidentalis* flit more in the presence of (*S*)-lavandulyl acetate than in the presence of (*R*)-lavandulyl acetate (Figure 4.12). Though, no valid conclusion can be drawn from this experiment due to the absence of a control disc (without synthetic compound), however, it shows that female *F. occidentalis* behaved differently with the two compounds.

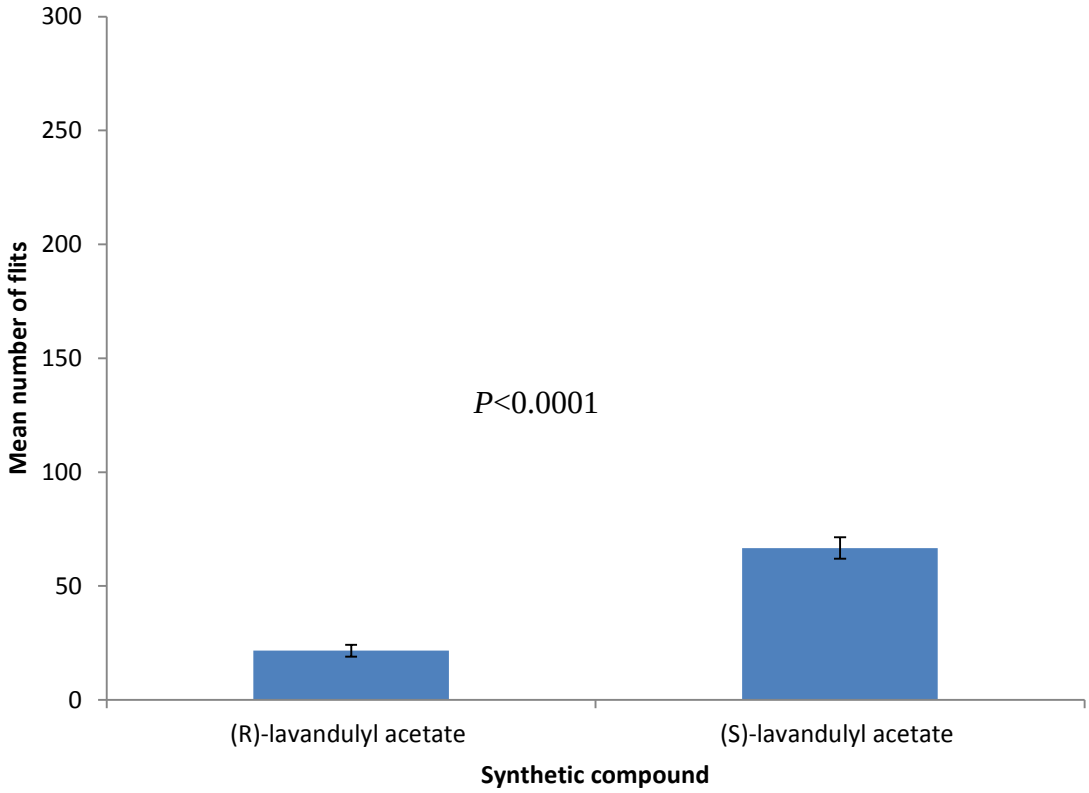

Figure 4.12 The mean (± SE) number of flits by adult female *F. occidentalis* on the filter disc during the no-choice bioassay period of 30 min. The filter paper discs were treated with (*R*)-lavandulyl acetate and (*S*)-lavandulyl acetate. There was a significant difference between the flit activity of adult female *F. occidentalis* on the (*R*)-lavandulyl acetate treated disc and (*S*)-lavandulyl acetate treated disc (ANOVA, $F_{1,23}$ = 70.54, P<0.0001).

4.3.6 Response of adult male *F. occidentalis* to (*R*)-lavandulyl acetate in a no-choice bioassay

To detect any behavioural differences between the sexes, adult male *F. occidentalis* was tested against (*R*)-lavandulyl acetate.

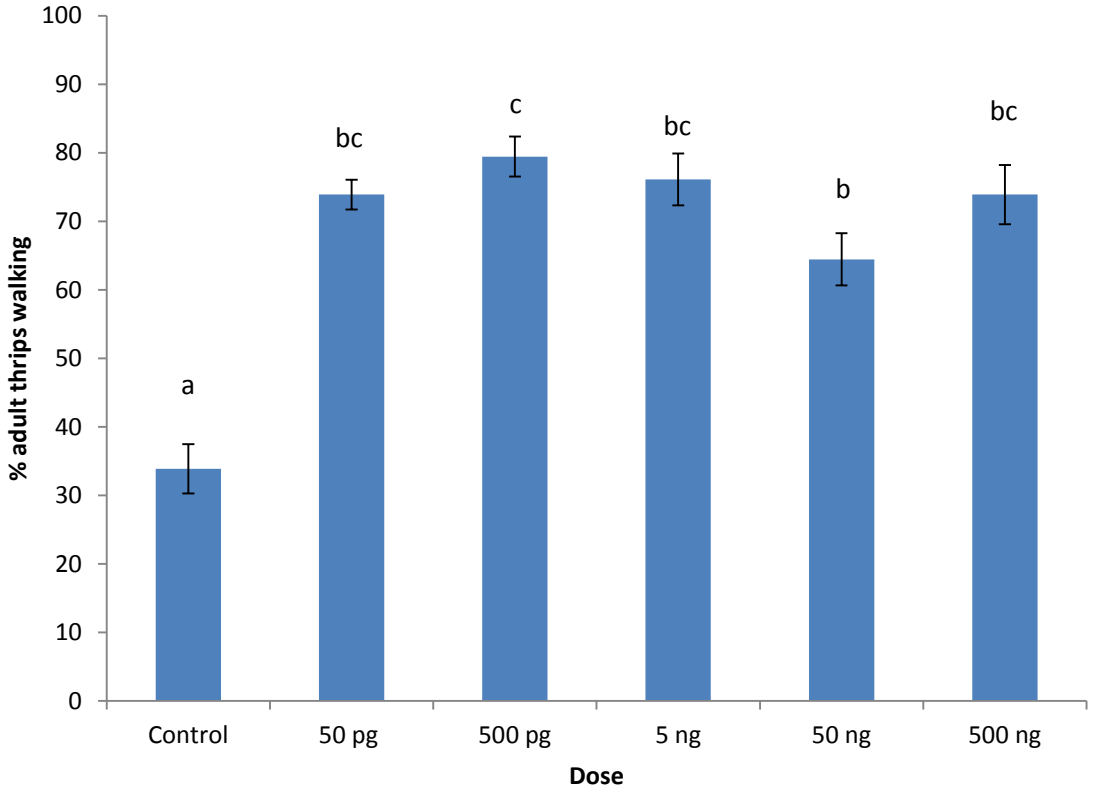

Figure 4.13 The mean (± SE) percentage of adult male *F. occidentalis* walking on the filter paper disc during the no-choice bioassay period of 30 min. The filter paper discs were treated with (*R*)-lavandulyl acetate. There was a significant difference between the walking activity of adult male *F. occidentalis* on the treated discs and the control disc (ANOVA, $F_{5,35} = 26.86$, $P < 0.0001$). n=12 trials.

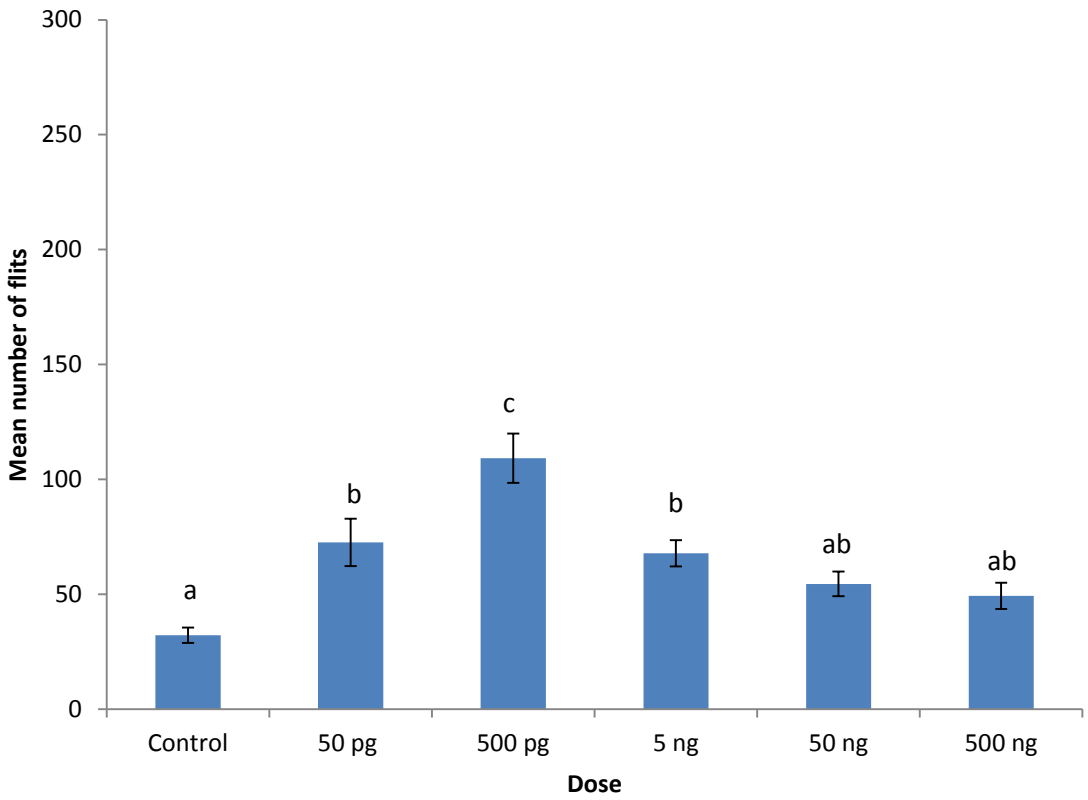

Figure 4.14 The mean (± SE) number of flits by adult male *F. occidentalis* on the filter disc during the no-choice bioassay period of 30 min. The filter paper discs were treated with (*R*)-lavandulyl acetate. There was a significant difference between the flit activity of adult male *F. occidentalis* on the treated discs and the control disc (ANOVA, $F_{5,35} = 16.94$, $P < 0.0001$). n=12 trials.

Adult male *F. occidentalis* walked more compared to the control disc (ANOVA, $F_{5,35} = 26.86$, $P < 0.0001$) suggesting a high level of activity (Figure 4.13). The lowest mean number of walking thrips was recorded on the control disc while 500 pg gave the highest mean number of walking thrips. The mean number of flits by adult male *F. occidentalis* was significantly different (ANOVA, $F_{5,35} = 16.94$, $P < 0.0001$). The highest flit was recorded with 500 pg while the control disc gave the lowest mean number of flits (Figure 4.14).

4.4 Discussion

The bioassay used has provided much needed information on the biological activities of adult female *F. occidentalis* when presented with both male-exposed discs (natural pheromone) and the identified male-produced compounds, neryl (*S*)-2-methylbutanoate and (*R*)-lavandulyl acetate (synthetic pheromone).

4.4.1 Male-exposed disc (natural pheromone) compared with neryl (*S*)-2-methylbutanoate (synthetic pheromone)

Adult female *F. occidentalis* responded positively to both the male-exposed discs and synthetic neryl (*S*)-2-methylbutanoate. The response index shows that adult female thrips can detect and respond to the active compounds on the male-exposed discs.

The activity level of the female thrips increased considerably in the presence of the male-produced compounds. They walk and flit more when compared with non-exposed discs proving further the presence of active male-produced compounds. This was expected as it has been found that adult male *F. occidentalis* produced two compounds (Hamilton *et al.*, 2005). A similar trend was observed with synthetic neryl (*S*)-2-methylbutanoate, adult female thrips responded to this compound. However, there was a difference in the response for both sources of pheromone, which simply implies that synthetic neryl (*S*)-2-methylbutanoate is not solely responsible for the observed behavioural responses exhibited by the adult female *F. occidentalis* when presented with the natural pheromone. The response index for the exposed discs could not be equaled by any of the doses of the synthetic compounds.

Furthermore, based on the estimated production rate of this compound (100–300 pg male^{-1} h^{-1}) (Dublon *et al.*, 2008), it was expected that the wide range of doses used in the bioassay should be able to capture the full effect if this compound is the sole cause of the response. However, combination of the two compounds may be another possible

explanation why such effects were not replicated. A mixture of the compounds varying their concentrations and ratio could account for the response or possibly other compounds may be involved. The possibility of additional compounds in the male-exposed disc was speculated upon by Dublon (2009). As it was reported in some species, there is the possibility of multiple roles with multiple male-produced compounds (Sirugue *et al.*, 2002), therefore is not unlikely that an additional compound may be responsible for the difference in the response or acting as a synergist with neryl (*S*)-2-methylbutanoate. Detection of significant effect across the doses used in the bioassay further shows that neryl (*S*)-2-methylbutanoate can be detected over a wide range. This suggests that the compound is a strong attractant as evidenced in the work of Brougton & Harrison (2012), Gomez et al. (2006) and Covaci et al. (2012). The result further suggests that neryl (*S*)-2-methylbutanoate was not only an attractant but also increased the activity level of female *F. occidentalis* which may be useful to activate thrips from their hidden places before chemical control is used.

4.4.2 Male-exposed disc (natural pheromone) compared with (*R*)-lavandulyl acetate (synthetic pheromone)

As observed with male-exposed filter paper discs, adult female *F. occidentalis* responded to (*R*)-lavandulyl acetate showing that the compound can be detected and responded to in a similar way to the other compound, neryl (*S*)-2-methylbutanoate. However, the response index was lower compared to that obtained with neryl (*S*)-2-methylbutanoate which suggests that (*R*)-lavandulyl acetate may be an attractant or arrestant at a short range at least for walking. The response index indicates a positive shift towards the compound which further suggests their preference for the treated disc. This may be one of the reasons for reduced thrips catch observed in the field with (*R*)-

lavandulyl acetate (Hamilton *et al.*, 2005). It is possible that thrips may not be landing on the traps but on the nearby plants thereby reducing the number recorded on the traps.

When the activity level was tested, adult female *F. occidentalis* behaved in a significantly different way to how they behaved with neryl (*S*)-2-methylbutanoate. The walking response showed a less mobile adult female thrips when presented with (*R*)-lavandulyl acetate. It suggests that most of the time during the experiment, they are stationary within the experimental arena. A similar result was recorded when the flit response was measured. All the doses tested were significantly lower than the control suggesting less active female thrips. These results suggest that (*R*)-lavandulyl acetate has a strong influence on the behaviour of adult female *F. occidentalis*; both flit and walking activities were affected by different doses used in this experiment.

The marked reduction in flits and walking of female *F. occidentalis* possibly indicate an arrestment behaviour which makes the female thrips immobile and stationary. It may be used during mating process when they are on the flower heads because it would have been expected that they will be active during feeding but less active during mating unless rejecting a mate. This further suggests that (*R*)-lavandulyl acetate is being used by adult male thrips to calm adult female *F. occidentalis* during mating. Pelikan (1951) suggests that adult male *Pezothrips dianthi* (=*Taeniothrips dianthi*) produces and deposits a compound on adult female *P. dianthi* which has a calming effect on the female. Therefore, (*R*)-lavandulyl acetate could be the calming compound produced by the male *F. occidentalis*. Comparing the differences in sexes, the result shows males are strongly active: they flit more and frequently walk round the arena. This may possibly have a role in the male-male fighting and consequently mating behaviour. Adult female *F. occidentalis* generally mate with the first male encountered and reject advances from other males; this

may be due to the (*R*)-lavandulyl acetate placed on the female/contacted by the male, thereby making them inactive and unreceptive to other males for a few days.

This result contradicts the conclusion of Zhu *et al*. (2012) that (*R*)-lavandulyl acetate is part of the aggregation pheromone of *F. occidentalis*. They concluded that a quantitative difference between the male-produced compounds of *F. occidentalis* and *F. intonsa* plays a role in interspecies identification. However, their ideas have not been tested with synthetic compounds. It is not clear how *F. occidentalis* and *F. intonsa* were able to distinguish between their odour since they produced the same compounds. This needs to be investigated though it has been established that *F. occidentalis* used another compound for species recognition (Olaniran *et al.*, 2013). Based on this work, the male-produced compounds seem to function differently and the ratio may not be a factor in their role. The field experiments with these compounds in different concentration and ratio did not show any increase in thrips catches in the field (Hamilton *et al.*, 2005) which suggests that ratio may not entirely be the reason for observed result in the field.

However, bearing in mind that most of the adult females used are likely to have mated, virgin female may behave differently to this compound. Due to time constraint, further experiments were not carried out as this may reveal other interesting information about the behavioural response of adult virgin and mated female thrips.

4.4.3 Detection of additional compound(s)

The results of synthetic pheromone bioassays have clearly shown that the responses obtained were unable to match those of the natural pheromone. Neryl (*S*)-2-methylbutanoate gave an indication of what was expected but not to the same extent as the responses from male-exposed discs. There were two main possibilities, the mixture of the two compounds at different concentrations and ratio may explain the effect or the presence of other compounds on the male-exposed disc may explain it. The possibility of other

compounds was explored by analyzing the compounds on a glass fibre disc provided by the Natural Resources Institute (NRI). The success with identifying another compound (see chapter 5) meant that the study of mixtures was not pursued.

4.5 References

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Chapter 5
Behavioural responses to 7-methyltricosane

5.1 Introduction

The behavioural response of *F. occidentalis* to male-exposed discs was unable to be replicated with synthetic compounds in the laboratory (see chapter 3). While neryl (*S*)-2-methylbutanoate attracts both female and male, the specific role of (*R*)-lavandulyl acetate is largely unknown (Hamilton *et al.*, 2005). The previous laboratory studies on these male produced compounds revealed that the outcome with male-exposed discs and synthetic compounds does not match up thereby raising a question of whether combination of the two compounds in different concentrations and ratio may reproduce the effect or additional compounds may be present on male *F. occidentalis*. Dublon (2009) suggested that the increased contact response observed in the filter paper disc choice bioassay may possibly be due to presence of a compound with low volatility and high molecular weight. Both sexes of *F. occidentalis* showed high contact response to filter paper discs exposed to live male *F. occidentalis* but this response was not observed with the use of synthetic male produced compounds, neryl (*S*)-2-methylbutanoate and racemic lavandulyl acetate. The contact response observed suggests that a low volatile, high molecular weight, cuticular compound may be involved.

Insect cuticle is coated with a complex mixture of long-chain fatty acids, alcohols, esters, aldehydes, ketones and hydrocarbons that primarily act as protectant against desiccation (Gibbs, 1998). Hydrocarbons have been widely studied for their complex role in chemical communication of insects. There is increasing evidence for their role as

pheromones, kairomones, recognition cues and for nestmate recognition (Howard & Blomquist, 2005). Cuticular hydrocarbons are sexually dimorphic in a wide range of species. Compounds present in one sex may be absent in the other, while compounds present in both sexes may differ quantitatively (Thomas & Simmons, 2008). In some species, age can affect the cuticular hydrocarbon; Chao *et al*. (2010) reported that male-specific cuticular compounds of *Drosophilla paulistorium* increased in quantity as adult flies aged. Social experience also influences these male-specific compounds, with socially-isolated flies having higher quantities than community raised ones (Kim *et al.*, 2004).

In Thysanoptera, cuticular hydrocarbon analysis of *F. occidentalis* has been reported by Golebiowski *et al*. (2007) and Zhao *et al*. (2011). They reported the results from analysis of adults and larvae without specifying the sex of the adults. However, it is likely that the analysis reported for the adults was mainly from females. This may arise due to the larger number of females in populations compared to males. The hydrocarbons reported were similar for both adults and larvae, though there were qualitative differences. High amount of 9-methylpentacosane was reported to be present in both adults and larvae (Golebiowski *et al.*, 2007) but was conspicuously absent in larvae analysed by Zhao *et al*. (2011). None of these hydrocarbons have been tested for their pheromonal activities in *F. occidentalis*.

Because of the observed contact response of *F. occidentalis* to naturally occurring pheromone and the evidence that the known male-produced compounds were not involved, it was suspected that an involatile compound may be responsible for this contact response.

5.1.1 Aims

This chapter attempts to test if there were any other unknown compounds produced by live male *F. occidentalis* as found on the male-exposed discs that could explain the

behavioural response of adult female *F. occidentalis.* This chapter also attempts to understand the specific role of the discovered compound by examining the walking and flitting behaviour of female thrips and the distance response of both female and male *F. occidentalis* to determine if they can detect and respond to the unidentified compound from a distance or need contact before responding to elucidate their role either as an attractant or otherwise.

5.2 Material and methods

5.2.1 Obtaining exposed discs for GC-MS

To collect the unknown compounds on male-exposed discs, glass microfiber filter paper discs were exposed to adult male *F. occidentalis* for 5 hours as described in section 3.2.2. Two samples were collected and these discs were exposed to 79 and 100 males respectively. Glass microfiber filters discs were used because they have a fine capillary structure and can absorb significantly larger quantities of chemical compounds than an equivalent cellulose filter. They can also be cleaned more effectively. Rolled glass microfiber filter paper discs (GF/A 24 mm circles; GE Healthcare UK Ltd, Bucks, UK) packed in stainless steel thermal desorption tubes and sealed with Swagelocks were sent from National Resources Institute (NRI). The rolled glass microfiber filter paper discs were carefully removed from the desorption tube, straightened and gently placed with hexane-cleaned forceps into the base of borosilicate glass tubes (height 20 mm x 30 mm diam.). Deionised water (15µl) was pipetted into the opposite side from the filter paper disc on the base of the borosilicate glass tubes (Digital 4 – 20µl, FinniPipette, Finland) to provide moisture to sustain the thrips. Mixed age adult thrips were aspirated from a white dish containing *F. occidentalis* as described in 3.2.2. The aspirator with thrips inside was placed on ice for 10 s and the sex determined under a dissecting microscope. Each aspirator vessel was gently removed from the aspirator and *F. occidentalis* were quickly

transferred by gentle tapping of the aspirator vessel into the glass tube with the filter paper disc. The control was left without live thrips. The glass tubes were covered with a stretched piece of Parafilm membrane (length 35 mm x 50 mm width) (Parafilm M, Pechiney Plastic Packaging, WI, USA). The glass tubes were placed on a horizontal rectangular pane of glass in the bioassay room as described in 3.2.2 to allow observation of the thrips underneath as well as above the filter paper discs. The exposure was carried out in the early hours of the day from 08:30 – 14:30 to follow the period already established that thrips produce pheromone in the laboratory (see chapter 3). The glass microfiber filter paper discs were removed from the glass tubes and carefully rolled back with the use of hexane-cleaned forceps into the stainless steel thermal desorption tubes. These were later sent to NRI for chromatographic analysis.

5.2.1.1 Collection and chemical analysis of cuticular hydrocarbons

Male, female and larvae samples were collected as described in 3.3.1 and placed in different glass vials (2 ml; Supelco). The samples were: 2 glass vials of 50 males each, 3 glass vials of 300 females each and 2 glass vials containing 300 second-instar larvae each. These were given to Dr Sudhakar Akella for chemical analysis. Each sample was extracted immediately with *n*-hexane (≥ 98% purity, Merck, Germany) for 5 min to obtain non-polar extracts, which were transferred to a clean glass vial and evaporated to 10 µl.

5.2.2 Sources of chemicals

Neryl (*S*)-2-methylbutanoate (98% purity, enantiomeric excess 98%) was synthesized by D. Hall (NRI) according to the method described by Hamilton *et al*. (2005), except that it was purified by silica gel column chromatography. 7-methyltricosane was synthesised and obtained from NRI. Tricosane (purity ≥ 99.5%, Fluka, Sigma-Aldrich, UK) was purchased from Sigma-Aldrich, UK, while *n*-hexane (≥ 98% purity, Merck, Germany) was purchased from Fisher Scientific, UK.

5.2.3 Choice bioassay of 7-methyltricosane: preparation and application

The filter paper disc-choice bioassay (see 3.2.3) was used to study the response of adult female *F. occidentalis* to synthetic compounds.

The treatment disc was injected with a 5 µl volume of synthetic compound in hexane and 5 µl volume of hexane was added to the corresponding control. The compounds were placed as one drop in the middle of the disc from where the solvent spread across the whole disc. The lids were added and Parafilm membrane was stretched round the edges of the Petri dish as described in 3.2.3.

A range of doses of 7-methyltricosane was used to detect if any responses were present when female *F. occidentalis* was introduced into the Petri dish arena containing the filter paper discs. The doses used were 50 pg (5 µl of 10 pg μl^{-1}), 500 pg (5 µl of 100 pg μl^{-1}), 5 ng (5 µl of 1 ng μl^{-1}), 50 ng (5 µl of 10 ng μl^{-1}) and 500 ng (5 µl of 100 ng μl^{-1}).

5.2.3.1 Observations: Choice bioassays

The Petri dish was divided into two equal halves using the treatment and control filter discs as the basis of division. The number of adult female thrips found on each half of the Petri dish was measured visually and recorded. Records were taken every 3 min for a total of 30 min giving 10 recordings per bioassay. However, other behavioural activities were observed and noted for any variations that may assist in the interpretation of the results.

5.2.4 No-choice bioassay of 7-methyltricosane: preparation and application

To investigate the activity level of adult female *F. occidentalis* to 7-methyltricosane, a no-choice bioassay was carried out as described in 4.2.4. The treatment disc was injected with 5 µl of synthetic compound in hexane and 5 µl of hexane was added

to the corresponding control. The lids were added and Parafilm membrane was stretched round the edges of the Petri dish as described in 3.2.3. This bioassay was carried out in the designated room as described in 4.2.1. The range of doses used was similar to that described in 5.2.3.

5.2.4.1 Observations: No-choice bioassays.

The number of thrips moving within the arena was recorded as a walking response and flight and landing was measured as a flit. These were recorded over a 30 min period. This was every 3 min for walking and continuously for flits. A walking response was recorded when a thrips walks within the arena over glass surface or filter paper disc. Flits were recorded as an event when a thrips leaves any part of the arena with an attempted flight/take off and landing. The same method was used as in section 4.2.7.

5.2.5 Choice bioassay of mixed-synthetic pheromone: preparation and application

Based on the identification of compounds on exposed discs, this bioassay was used to test if the mixed-synthetic pheromone was responsible for the behavioural response of female *F. occidentalis* recorded with male-exposed discs. The mixed-synthetic was prepared using the amount of compounds found on exposed discs (Table 5.1). The compounds were neryl (*S*)-2-methylbutanoate (8 ng), (*R*)-lavandulyl acetate (1 ng) and 7-methyltricosane (15 ng) in *n*-hexane (≥ 98% purity, Merck, Germany. This amount was used without scaling it down to the amount proportional to 15 male-exposed discs (see Chapter 4), because more thrips does not always result in more pheromone is being produced. Density has been found to affect the activity level of *F. occidentalis* (see Chapter 7) which presumably may affect the rate of pheromone production. This assay follows the similar procedure described in 4.2.3. The treatment was mixed-synthetic pheromone in hexane while hexane was the control.

5.2.5.1 Observations: Choice bioassays

The Petri dish was divided into two equal halves using the treatment and control filter discs as the basis of division. The number of adult female thrips found on each half of the Petri dish was measured visually and recorded. Records were taken every 3 min for a total of 30 min giving 10 recordings per bioassay. However, other behavioural activities were observed and noted for any variations that may assist in the interpretation of the results.

5.2.6 No-choice bioassay of mixed-synthetic pheromone: preparation and application

To test whether the observed walking and flitting activity of adult female *F. occidentalis* recorded with male-exposed discs can be explained by using the mixed-synthetic compounds on an artificial substrate. The mixed-synthetic was prepared as described in section 5.2. 5. The bioassay was done as described in 4.2.4.

5.2.6.1 Observations: No-choice bioassays.

The number of thrips moving within the arena was recorded as a walking response and flight and landing was measured as a flit. These were recorded over a 30 min period. This was every 3 min for walking and continuously for flits. A walking response was recorded when a thrips walks within the arena over glass surface or filter paper disc. Flits were recorded as an event when a thrips leaves any part of the arena with an attempted flight/take off and landing.

5.2.7 Distance response bioassay

To test if thrips were responding to the compound from a distance or only after they touch the disc containing the compound, synthetic compounds were tested against adult male and female *F. occidentalis*. A set of 100 mm diameter glass Petri dishes and their lids (Anumbra, Scientific Glass Laboratories Limited, Tunstall, UK) were cleaned and

prepared as previously described in section 2.5. Two equidistant positions, 20 mm from the centre of the dish to both sides of a middle line dividing the Petri dish into equal halves, were marked out on the outer base of the Petri dish in order to position the filter discs without any bias to give 40 mm as the distance between the nearest edges of the filter paper discs. Cellulose filter discs (Whatman International Limited (1001020) grade 1, 20 mm diam.) were placed on the two positions marked on the Petri dishes using forceps cleaned with hexane (n-hexane, pesticide residue analysis grade (1526764), VWR International Limited, Poole, UK).

The treatment disc was injected with 5 µl of synthetic compound and the same volume of *n*-hexane was injected to the corresponding control. This was left for 2 min to allow hexane to evaporate before the introduction of thrips. Individual adult *F. occidentalis* were transferred to the middle of the Petri dish containing the treatment and the control. Treatment and control filter disc positions were reversed after each thrips response. After five responses, filter paper discs and Petri dish were replaced with new sets. This was carried out between the hours of 12:00 and 16:00 h in the bioassay room described in 3.2.2. The time period was chosen to have a consistent bioassay as it was already established that thrips responded during that time in previous laboratory bioassays. The compounds tested were: 200 pg 7-methyltricosane (synthesised by NRI) in 5 µl *n*-hexane (≥ 98% purity, Merck, Germany); 200 pg neryl (*S*)-2-methylbutanoate (synthesised by NRI) in 5 µl *n*-hexane; and 5 µl *n*-hexane. The dose (200 pg) was chosen to reflect the amount of 7-methyltricosane found on the thrips cuticle (see 5.3.1.1) while neryl (*S*)-2-methylbutanoate was to serve as a positive control to detect any behavioural response specific to a known attractant.

5.2.7.1 Observations: Distance bioassay

Each individual was observed for 3 min to see which of the two discs was touched first by walking. If within 3 min, the thrips did not touch the discs, it was regarded as a non-responder. This was recorded to obtain twenty responses; non-responders were not used in the analysis. However, non-responders were infrequent in the bioassay.

5.2.8 Contact response bioassay

To test the effect of 7-methyltricosane, and two controls, *n*-tricosane and hexane on the contact response of *F. occidentalis*, a glass bead bioassay was used. Glass beads were used to serve as dummy thrips because of their small size and because they can be cleaned. The bioassay was performed with synthetic compounds that were applied to glass beads (diam. 1 mm, Thistle Scientific Ltd. Glasgow, UK). A single glass bead was gently placed into the middle of a 40 mm Petri dish arena with 5 mm zone marked round the glass bead. It was treated with 1 µl volume (200 pg) of the synthetic compounds to be tested 3 min before the trial started to allow evaporation of the solvent. The amount of solvent (1 µl) was selected because it just coated the bead. A test thrips was transferred into the marked zone area containing the treated glass bead and the dish was gently covered with the lid. This dish arena was placed on a rectangular shelf, to allow observation of thrips, in the bioassay room as described above for collection of compounds on exposed discs.

5.2.8.1 Observations: Contact bioassay

The response of test thrips was observed by eye. The behaviour of each thrips was recorded for 3 min following when it first touched the glass bead. The abdomen raising was recorded when it is raised above 45º to horizontal and wagging at an angle of at least 20º to the side. The time taken to leave the 5 mm zone was recorded with the number of times the abdomen was raised by females and wagging to the side by males. However, if a test thrips did not touch the glass bead within 2 min, it was regarded as a non-responder

and therefore not used in the analysis. The total number of thrips used in the analysis was 20 for each sex.

5.2.9 Statistical analysis

5.2.9.1 Choice bioassays

The number of adult female thrips found on each half of the Petri dish was measured visually and recorded. Data collected were averaged for each bioassay and processed to obtain a Response Index (RI) as described in 3.3.4. The RI was analysed using General Linear Model ANOVA, Minitab 16 (GLM) and all data were tested to confirm normality as described in 2.7.

5.2.9.2 No-choice bioassays

The walking and flit responses of female western flower thrips were recorded as described in 4.2.7. The walking response data was analysed using General Linear Model ANOVA and Tukey's test was used to compare walking between treatments. However, flits data were not normally distributed due to the presence of unusually low and high values. Log transformation was then applied, the transformed and normalised data were then analysed using General Linear Model, Minitab 16, ANOVA and Tukey's test was used to compare between treatments and control.

5.2.9.3 Distance bioassay

The choices made were analysed using a two-tailed binomial test with exact probabilities and computed with IBM SPSS statistics version 19 and the Exact Tests module (IBM Corporation, USA), which allows for sparse data.

5.2.9.4 Contact bioassay

The amount of time spent within the 5 mm zone, the number of times the abdomen was raised (females) and the amount of abdominal wagging (males) were all analysed

using Kruskal-Wallis tests. The data were not normally distributed due to high and low values. Multiple comparisons were done using a *post-hoc* test, Holms's adjusted *P*. The 95% confidence interval of the median was obtained with Minitab 16.

5.3 Results

5.3.1 Identification of compounds on exposed discs

Chromatography analysis was done at NRI by Prof. David Hall. His results are summarized by him in the following paragraph.

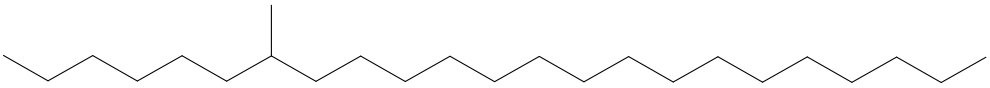

Figure 5.1: Chemical structure of 7-methyltricosane

Table 5.1 Male-specific compounds present on glass microfiber disks exposed to adult male *F. occidentalis* for 5 h. These data were provided by D. Hall (NRI).

Compound	GC Retention Index		Amount (range) [a]
	Non-polar DB5	Polar DBWax	(pg male^{-1})
Lavandulyl acetate	1287	1600	12 (10-13)
Neryl 2-methylbutanoate	1573	1859	89 (85-93)
7-Methyltricosane	2343	2332	175 (111-240)

[a] Mean of two collections

Extracts of glass microfiber discs exposed to adult male or female *F. occidentalis* were analysed by GC-MS and GC-FID on non-polar columns under conditions that would elute *n*-alkanes up to 38-carbons. Compounds detected in extracts of the discs exposed to males and not in those exposed to females were identified by their GC retention indices and mass spectra. These were decyl and dodecyl acetate, lavandulyl acetate, neryl 2-methyl butanoate and an unknown compound at retention index (RI) 2343. The mass spectrum of

the latter indicated it was a long-chain hydrocarbon with ions at *m/z* 112 and 252 suggesting it was 7-methyltricosane. The same compounds were observed in GC-MS analyses on a polar GC column. Retention indices of these compounds and the approximate amounts present on the discs exposed to male *F. occidentalis* are given in Table 5.1.

5.3.1.1 Analysis of cuticular hydrocarbons

Based on the evidence that an additional compound or compounds may be present in male *F. occidentalis*, I discussed the idea with Dr Akella and we both agreed to conduct the experiment. However, the GC (HP 5890 II +)-MS (HP 5972A) model we had at that time in the chemical ecology laboratory was not sensitive enough to detect many compounds. Dr Sudhakar Akella then collaborated with Dr Falko Drijfhout (School of Physical and Geographical Sciences at Keele University) to complete the GC-MS analysis. The extracts obtained were analysed on an HP7890 GC system coupled to a HP5975 Network Mass Selective Detector (Agilent).

The results presented show that adult *F. occidentalis* hydrocarbons consist of a mixture of *n*-alkanes, branched monomethyl alkanes and branched dimethyl alkanes. Long-chain, methyl-branched and dimethyl-branched hydrocarbons are more abundantly present in adults, whereas the larval extracts consist primarily of *n*-alkanes. Further analysis of cuticular hydrocarbons revealed large amounts of 7-methyltricosane on males, whereas only trace amounts were found on females and none on larvae. The concentrations in the two male samples were 160 pg male^{-1} and 236 pg male^{-1}, which gives a mean of 198±38 pg male^{-1}. However, there were two additional compounds present in comparatively higher concentrations in males of *F. occidentalis* than in females. These compounds were 9-methylpentacosane and 7-methylpentacosane.

5.3.2 Response of adult female *F. occidentalis* to 7-methyltricosane in a choice bioassay

Adult female *F. occidentalis* showed no significant response to the disc with 7-methyltricosane at all doses (Table 5.2). The result showed a trend towards positive preference for the treated disc. When all the RI (n=60) were tested together, the result was significant (Table 5.2). The result showed a weak preference for the compound. There was no significant difference in response across the doses (ANOVA, $F_{4,59}$ =0.30, P=0.875) (Figure 5.2).

Table 5.2 The mean response index (RI) of adult female *F. occidentalis* to 7-methyltricosane and hexane-control discs in a choice bioassay. The *P*-values for a one-sample *t*-test of the difference from zero is given. n=12 trials.

Dose	Mean	SEM	*P* value
50 pg	0.0117	0.0097	0.253
500 pg	0.0125	0.0118	0.313
5 ng	0.0267	0.0139	0.081
50 ng	0.0125	0.0129	0.354
500 ng	0.0133	0.0081	0.151
All*	0.015	0.0054	0.004

* The analysis of the RI pooled together to have a total of 60.

5.3.3 Response of adult female *F. occidentalis* to 7-methyltricosane in a no-choice bioassay

The mean number of thrips walking was significantly different across the treatments (ANOVA, $F_{5,35}$ =8.13, P<0.0001) (Figure 5.3). The mean numbers of adult female thrips walking in all the five doses were not significantly different from one another but were different from the number walking in the control. The mean number of flits was significantly different for all the tested treatments (ANOVA, $F_{5,35}$ =3.64, P=0.013). The

mean number of thrips for 5 ng and 50 ng were significantly higher than the number of flits recorded for the control disc suggesting the doses at which the compound is most effective (Figure 5.4)

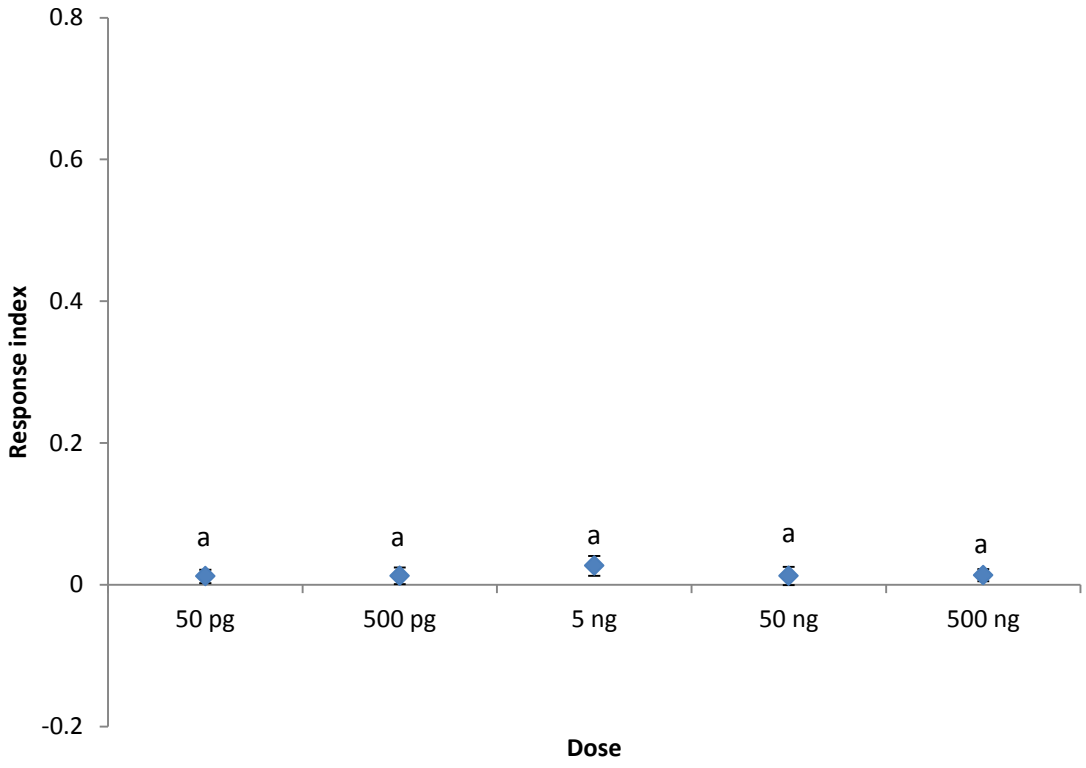

Figure 5.3 Filter paper disc choice bioassay. Mean response index (± SE) of mixed-age adult female *F. occidentalis* to discs treated with different doses of 7-methyltricosane. Response index was calculated as (T - C)/(T + C), where T is the number of test thrips in treatment half (treated-disc) and C is the number of test thrips present in the control half (blank disc). Means with the same letter are not significantly different at P<0.05 (Tukey), n= 12 trials.

5.3.4 Response of adult female *F. occidentalis* to mixed-synthetic pheromone in a choice bioassay

The response index (0.566 ± 0.044) of adult female *F. occidentalis* to mixed-synthetic pheromone was significantly higher than zero ($t_{(11)} = 12.81$, $P < 0.0001$). As

mentioned in 4.3.1, it shows that adult female *F. occidentalis* preferred the arena side with mixed-synthetic pheromone and they can respond to the mixture. Unfortunately, there was no male-exposed discs as a control or comparisons with neryl (*S*)-2-methylbutanoate, but the RI is higher than in any previous experiment with synthetic compounds.

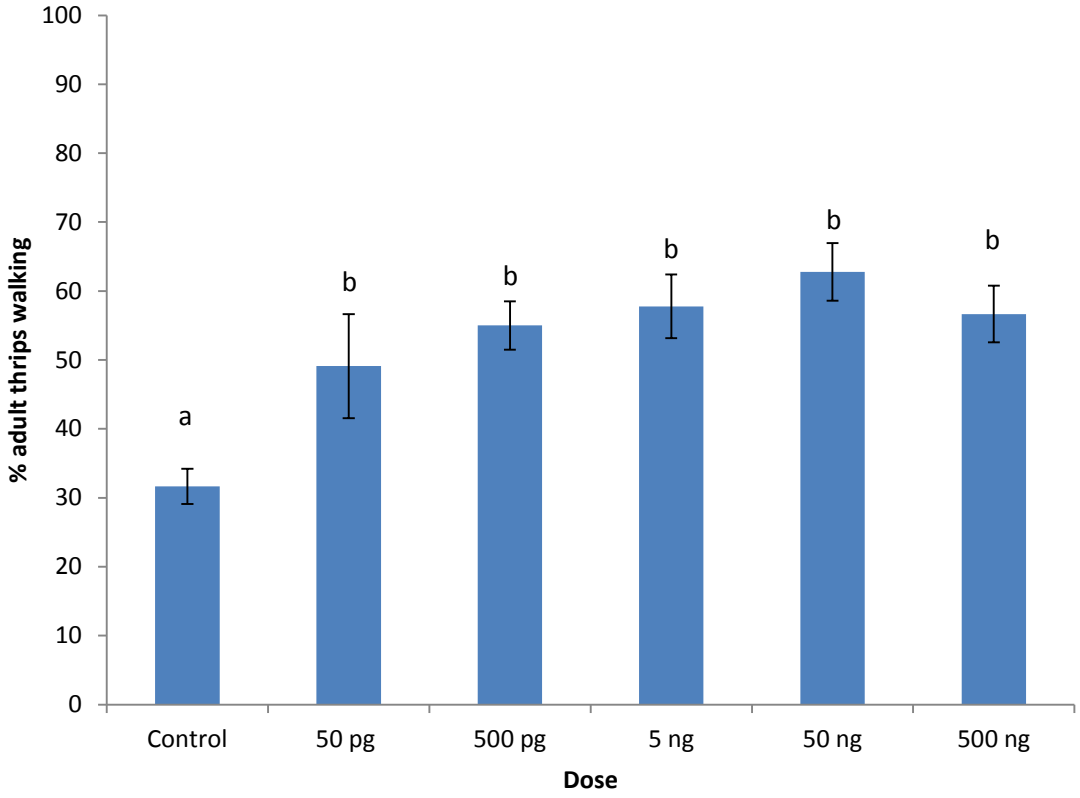

Figure 5.3 The mean (± SE of the mean) percentage of adult female *F. occidentalis* walking during the no-choice bioassay period of 30 min. The filter paper discs were injected with 7-methyltricosane. There was significantly more walking activity of adult female *F. occidentalis* (ANOVA, $F_{5,35}$ = 8.13, P<0.0001). n=12 trials. Means with different letters indicate significance at P<0.05 (Tukey).

5.3.5 Response of adult female *F. occidentalis* to mixed-synthetic pheromone in a no-choice bioassay

As explained in 4.3.4, walking and flit responses were used to assess the behavioural response of adult female *F. occidentalis* in a no-choice bioassay. The mean

number of adult female thrips walking was not significantly different between synthetic-mixed pheromone and hexane control disc (ANOVA, $F_{1,29}$ =2.83, P=0.104) (Figure 5.5). However, the flit response of adult female *F. occidentalis* to synthetic-mixed was significantly higher than the hexane-control disc (ANOVA, $F_{1,29}$ =6.12, P=0.02). (Figure 5.6).

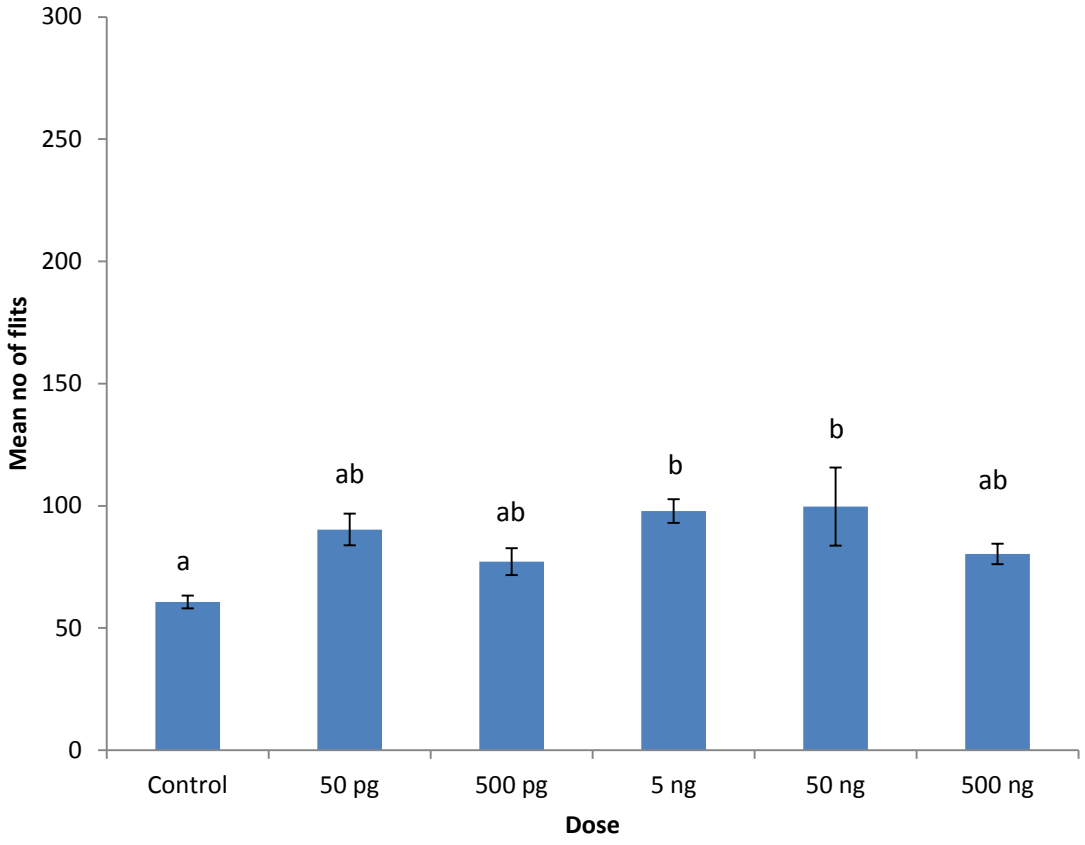

Figure 5.4 The mean (± SE of the mean) number of flits by adult female *F. occidentalis* on the filter disc during the no-choice bioassay period of 30 min. The filter paper discs were treated with 7-methyltricosane. There was significantly more flit activity of adult female *F. occidentalis* on the treated discs than on the control disc (ANOVA, $F_{5,35}$ = 3.64, P= 0.013). n=12 trials. Means with different letters indicate significance at P<0.05 (Tukey).

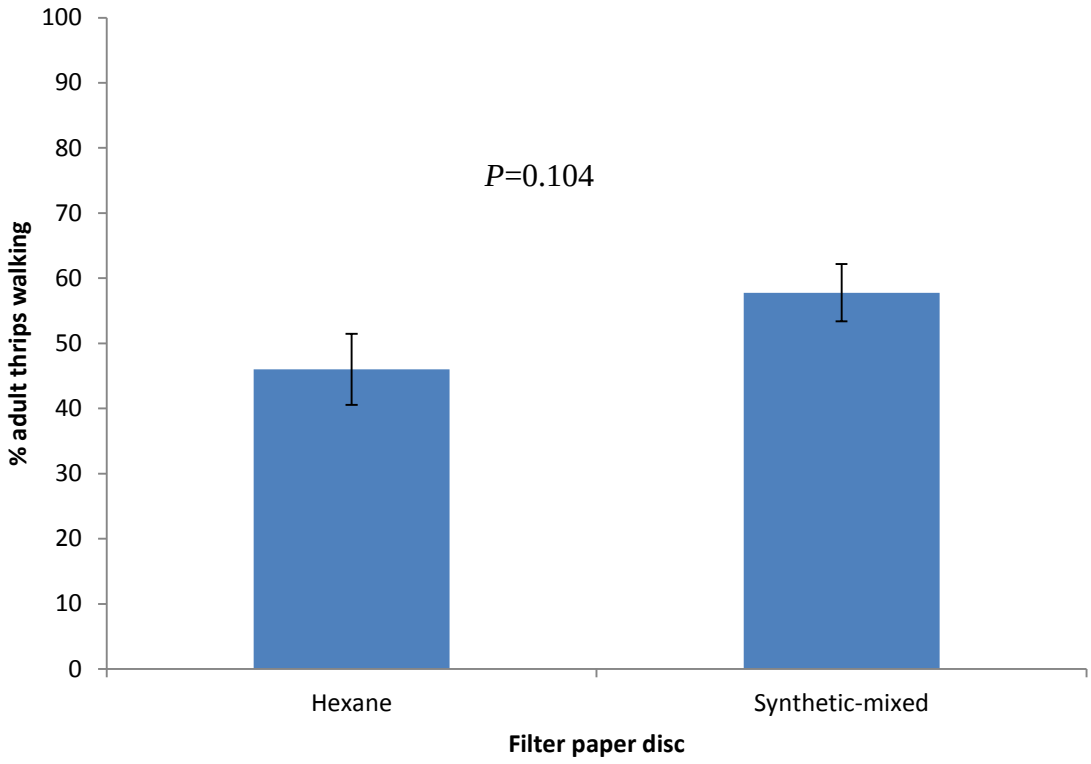

Figure 5.5 The mean (± SE of the mean) percentage of adult female *F. occidentalis* walking on the filter paper disc during the no-choice bioassay period of 30 min. The filter paper discs were injected with synthetic-mixed compound containing neryl (*S*)-2-methylbutanoate, (*R*)-lavandulyl acetate and 7-methyltricosane. There was no significant difference between the walking activity of adult female *F. occidentalis* on the treated discs and the control disc (ANOVA, $F_{1,29}$ = 2.83, *P*= 0.104). n=12 trials.

5.3.6 Distance response bioassay to synthetic pheromone

To test whether 7-methyltricosane can attract from a distance without contact, a known attractant, neryl (*S*)-2-methylbutanoate was tested against hexane to serve as a

positive control. This was done to help determine whether 7-methyltricosane is a contact pheromone or otherwise.

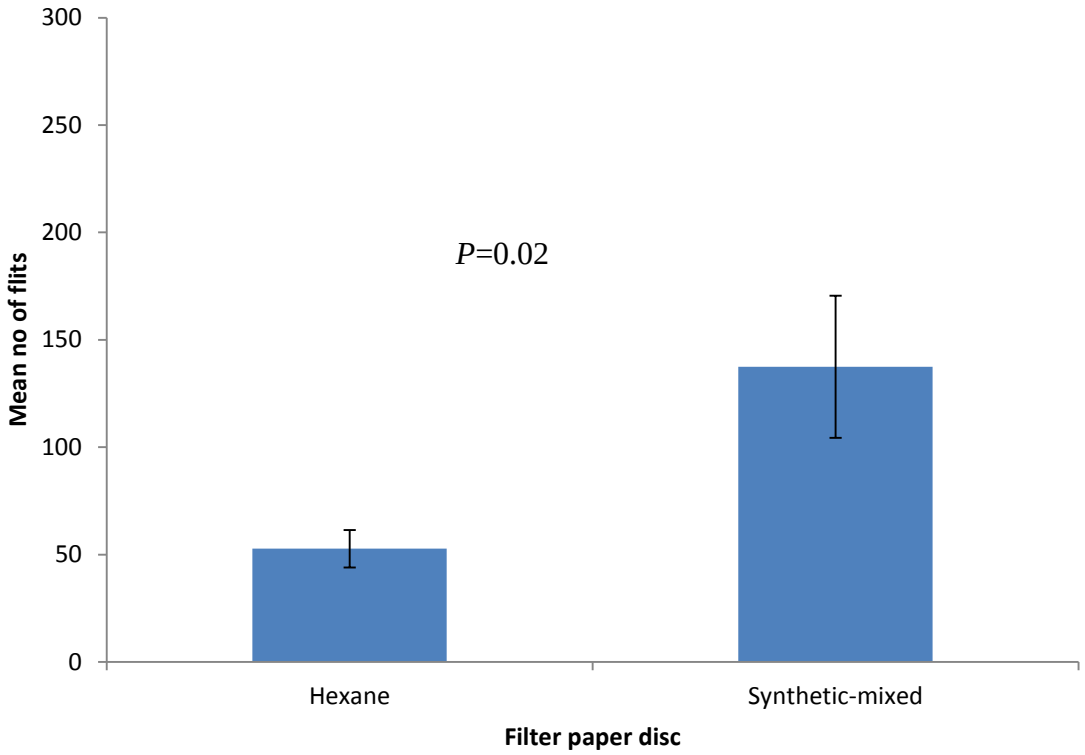

Figure 5.6 The mean (± SE of the mean) number of flits by adult female *F. occidentalis* on the filter disc during the no-choice bioassay period of 30 min. The filter paper discs were treated with synthetic-mixed pheromone containing neryl (*S*)-2-methylbutanoate, (*R*)-lavandulyl acetate and 7-methyltricosane. There was significantly more flit activity of adult female *F. occidentalis* on the treated discs compared to the control disc (ANOVA, $F_{1,29} = 6.12$, $P = 0.02$). n=12 trials.

It was expected that if it is a contact pheromone the thrips will touch the treated disc as often as the control disc because they cannot detect it from a distance. The positive control experiment showed a distinct response. Adult female *F. occidentalis* chose the treatment side 23 times while the hexane control side was chosen 7 times (binomial test, $n = 30$, $P=0.005$). Adult males went to the treatment side 25 times while only 5 responses were

recorded for the hexane control (binomial test, n= 30, P<0.001). This significantly confirmed that the bioassay can be used to detect responses of *F. occidentalis* from a distance of about 20 mm. This bioassay was then used to test the responses of *F. occidentalis* to 7-methyltricosane and the hexane control. Adult females went to the treatment side 16 times while 14 was recorded for the hexane (binomial test, n= 30, P=0.86) and males had 13 treatment choices and 17 control choices (binomial test, n= 30, P=0.58). This clearly reveals that in this bioassay *F. occidentalis* cannot detect 7-methyltricosane from a distance of a few millimetres at a dose equivalent to the amount found on individual males (Table 5.3).

The mean time taken to choose (that is reach either disc) in the positive control was significantly lower for the test compound compared with the control for both females (ANOVA, $F_{1,29}$ =59.80, P<0.001) (Figure 5.7) and males (ANOVA, $F_{1,29}$ =38.77, P<0.001) (Figure 5.8). In contrast, there was no significance difference in mean time taken to choose between 7-methyltricosane and hexane-control by adult females (ANOVA, $F_{1,29}$ =0.33, P=0.571) (Figure 5.9) and males (ANOVA, $F_{1,29}$ =0.37, P=0.546) (Figure 5.10). The mean shortest time was recorded by males in the presence of the known attractant while the time taken by both sexes were similar in the presence of 7-methyltricosane. These results confirmed the positive response from a distance to neryl (*S*)-2-methylbutanoate and not to 7-methyltricosane.

Table 5.3 Responses from a distance of adult males and females of *F. occidentalis* to neryl (*S*)-2-methylbutanoate, 7-methyltricosane and hexane-control in a filter paper disc bioassay.

	Treatment	Control	
Neryl (*S*)-2-methylbutanoate			
Females	23	7	*P*=0.005
Males	25	5	*P*<0.0001
7-methyltricosane			
Females	16	14	*P*=0.856
Males	13	17	*P*=0.585

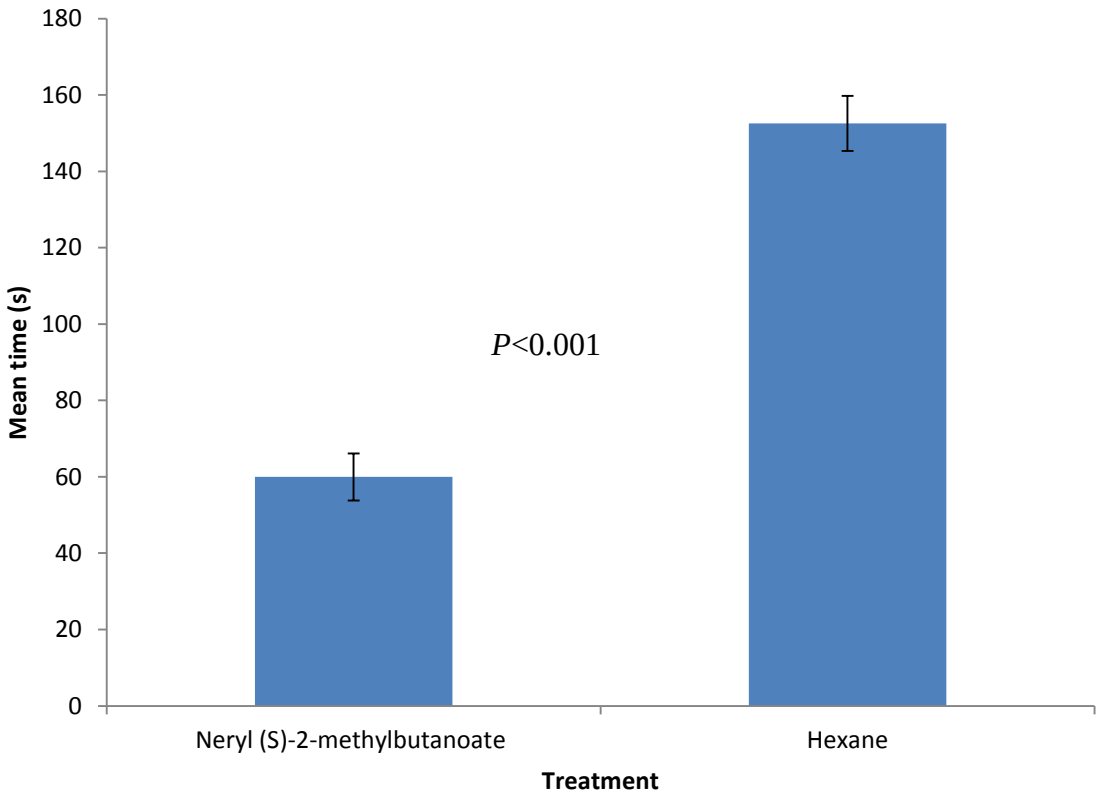

Figure 5.7 The mean (± SE of the mean) time used by adult females *F. occidentalis* to make a choice between neryl (*S*)-2-methylbutanoate and hexane in a distance bioassay, n=20 trials.

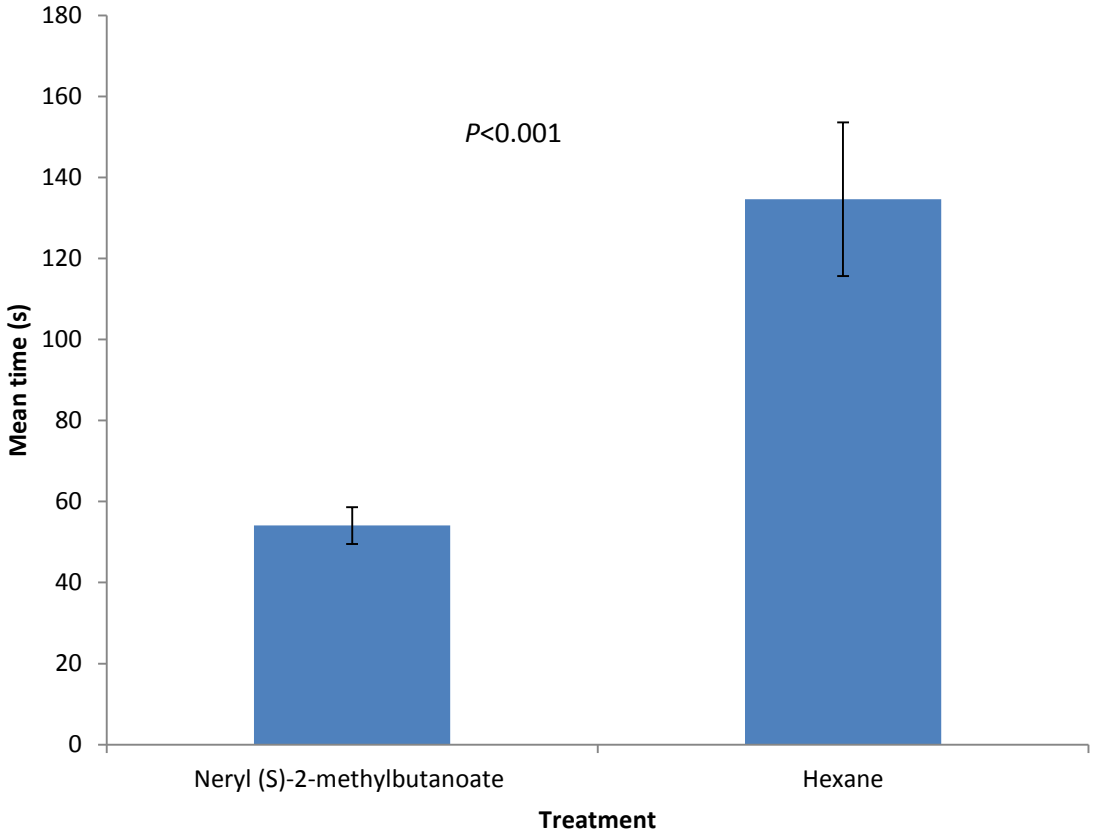

Figure 5.8 The mean (± SE of the mean) time used by adult males *F. occidentalis* to make a choice between neryl (*S*)-2-methylbutanoate and hexane in a distance bioassay, n=20 trials.

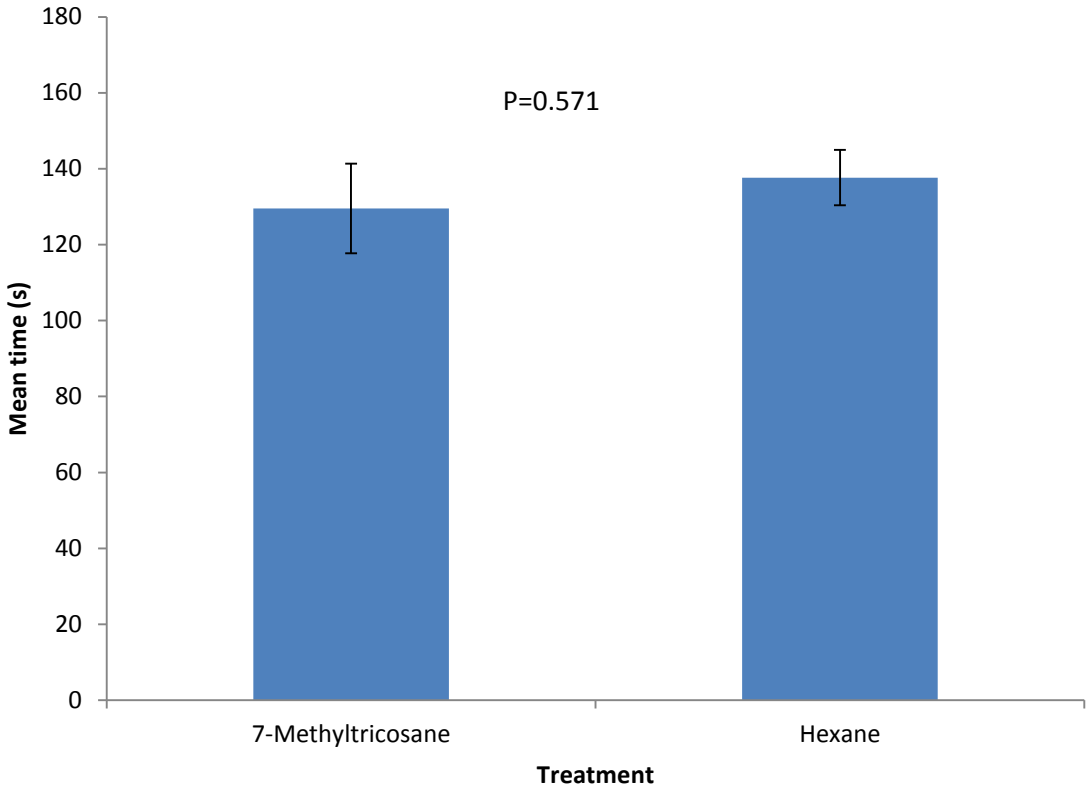

Figure 5.9 The mean (± SE of the mean) time used by adult males *F. occidentalis* to make a choice between 7-methyltricosane and hexane in a distance bioassay, n=20 trials.

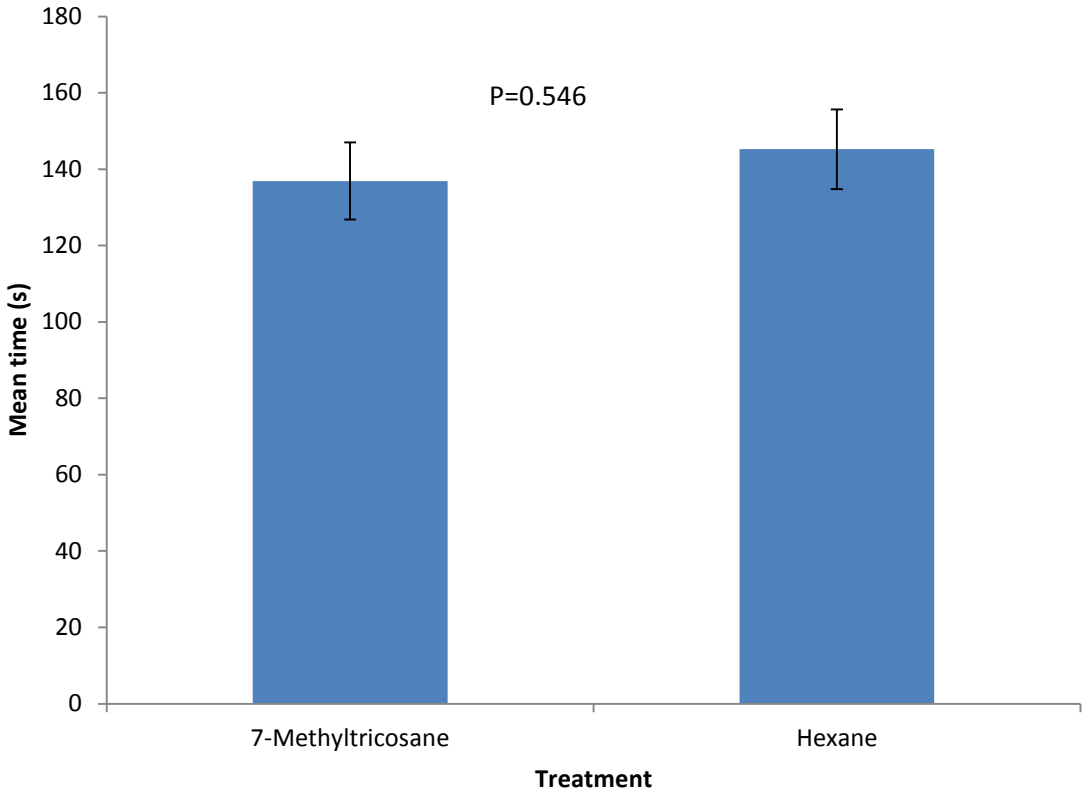

Figure 5.10 The mean (± SE of the mean) time used by adult females *F. occidentalis* to make a choice between 7-methyltricosane and hexane in a distance bioassay, n=20 trials.

5.3.7 Contact response bioassay to synthetic pheromone

The response to synthetic 7-methyltricosane was observed by coating a thrips-sized glass bead (diam. 1 mm) with an amount of compound likely to be on one individual male (200 pg). The responses were compared to hexane-only and tricosane controls. Tricosane occurs in much lower quantity in females, males and larvae. The amount of time spent in the marked zone of the coated glass bead was recorded. Adult females (Kruskal-Wallis, H= 41.78, *df*=2, *P*<0.001) and males (Kruskal-Wallis, H= 35.68, *df*=2, *P*<0.001) stayed longer in the marked zone of glass beads treated with 7-methyltricosane than in the zone of beads treated with tricosane or hexane-only (Fig. 5.11 and Fig. 5.12).

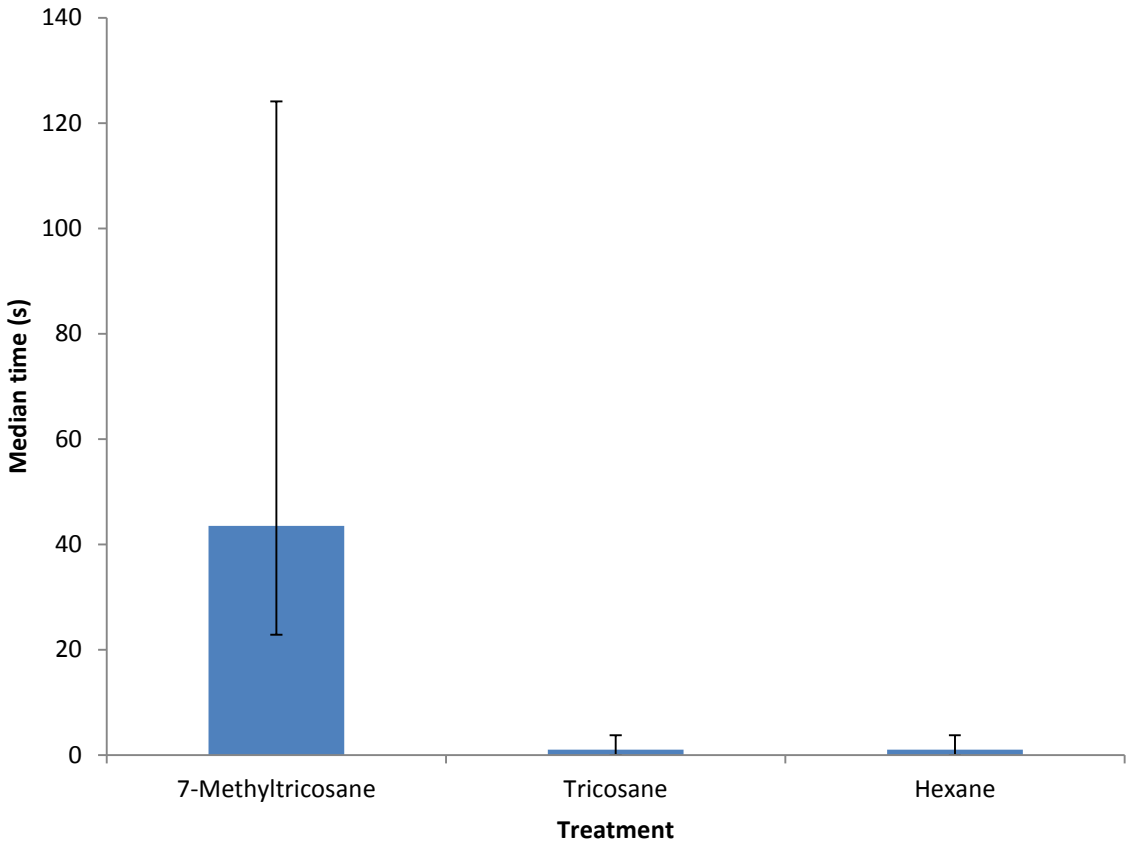

Figure 5.11 The median (± SE of the median) time spent in the marked zone by adult females *F. occidentalis* to synthetic pheromone (7-methyltricosane) and two controls (tricosane and a solvent-only blank) after contact with it on glass beads. (Kruskal-Wallis, H= 41.78, *df*=2, *P*<0.001)

Preliminary observations of responses to 7-methyltricosane revealed some distinctive behaviour associated with both sexes. Most times, females raised their abdomen at an angle approaching 90° to the substrate (Figure 5.13), a behaviour associated with females rejecting mating attempts from males, and males frequently arched the abdomen and wagged it from side to side, a behaviour observed during male-male interactions.

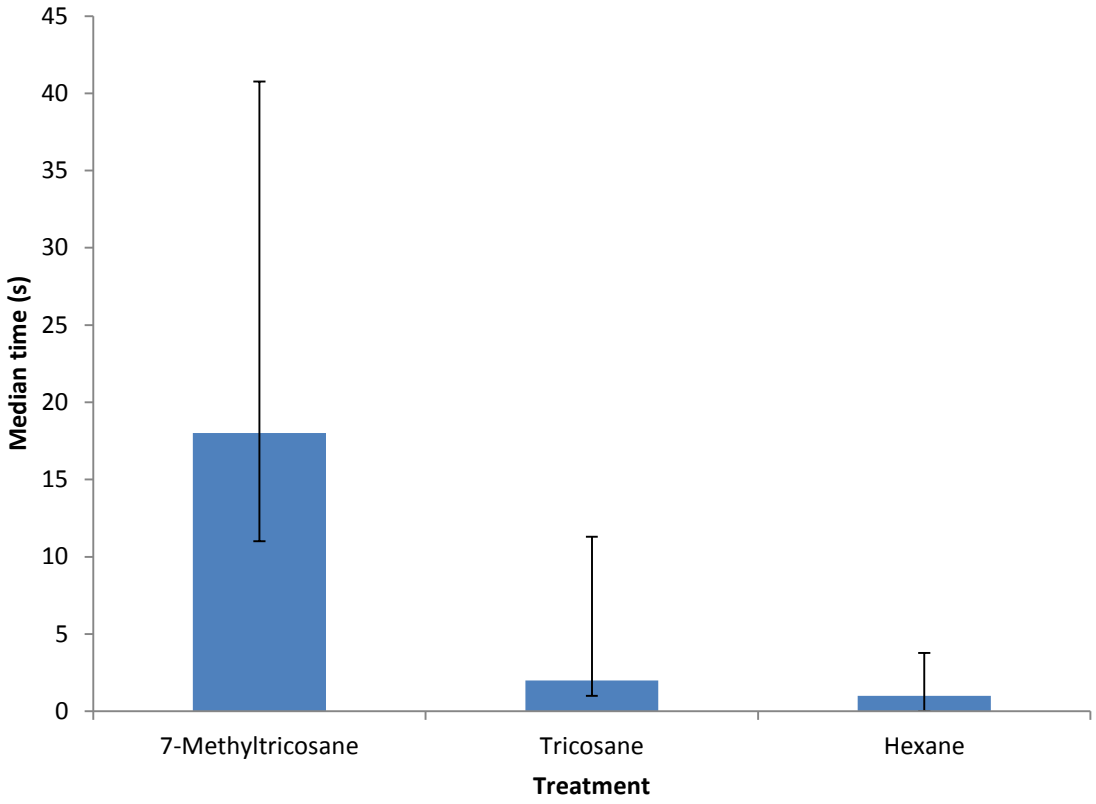

Figure 5.12 The median (± SE of the median) time spent in the marked zone by adult males *F. occidentalis* to synthetic pheromone (7-methyltricosane) and two controls (tricosane and a solvent-only blank) after contact with it on glass beads (Kruskal-Wallis, H= 35.68, *df*=2, *P*<0.001) n= 12 trials.

Adult females raised their abdomen more often after contact with 7-methyltricosane than after contact with *n*-tricosane or a hexane-only control (Kruskal-Wallis, H= 32.40, *df*=2, *P*<0.001) (Figure 5.14). However, some females were observed curling their abdomen round the glass beads; it is suspected that those females may have been virgin and ready to mate. It is not unlikely that adult females may be responding to the circular shape of glass beads but this was not frequent in the two controls. Adult males wagged their abdomen

more often after contact with 7-methyltricosane than after contact with *n*-tricosane or a hexane-only control (Kruskal-Wallis, H= 19.38, *df*=2, *P*<0.001) (Figure 5.15).

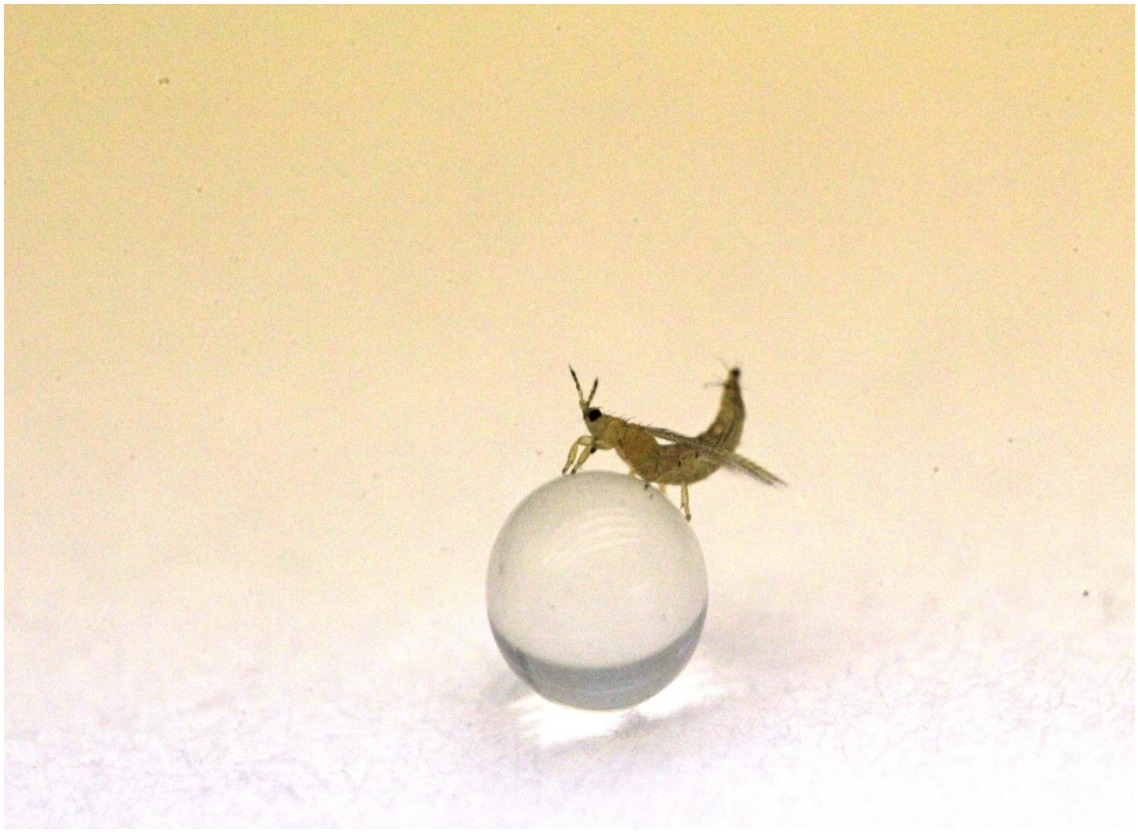

Figure 5.13 Photo of an adult female *F. occidentalis* responding to 7-methyltricosane on a glass bead by raising its abdomen, which is a behavior used to repel male mating attempts

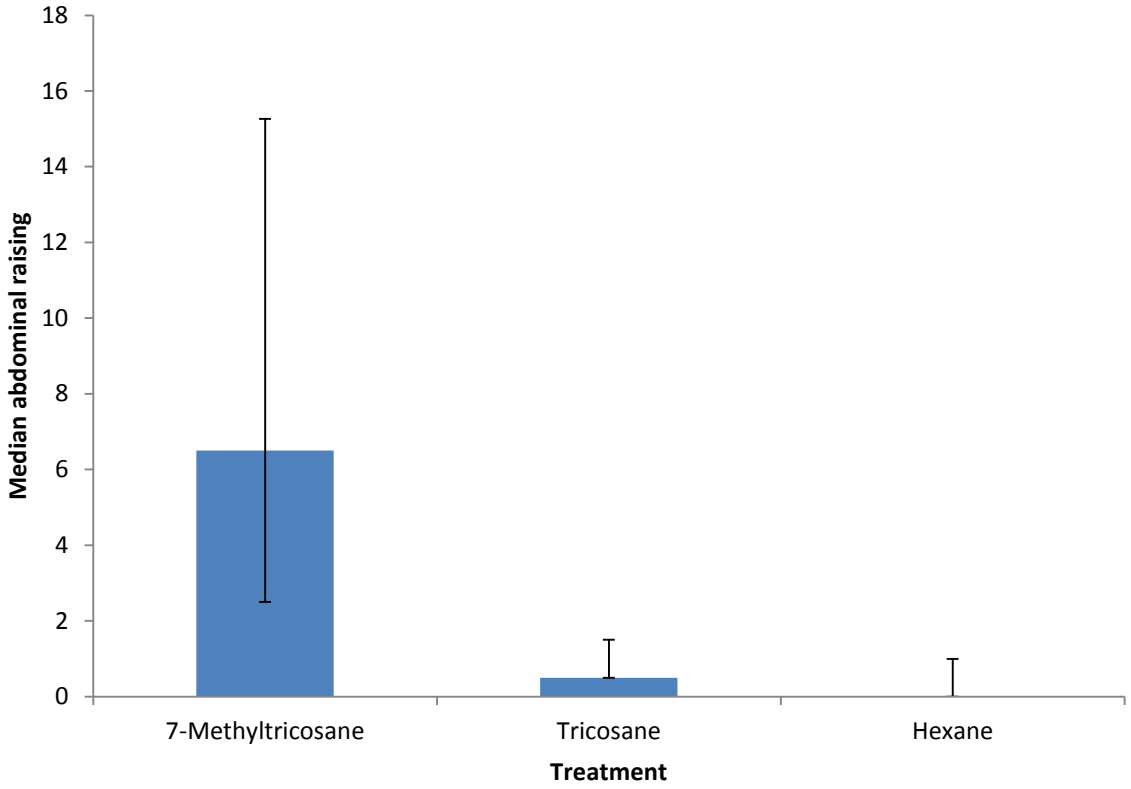

Figure 5.14 The median (± SE of the median) number of times adult female *F. occidentalis* raised the abdomen in response to synthetic pheromone (7-methyltricosane) and two controls (tricosane and a solvent-only blank) for 3 min after contact with it on glass beads (Kruskal-Wallis, H= 32.40, *df*=2, *P*<0.001) n=12 trials.

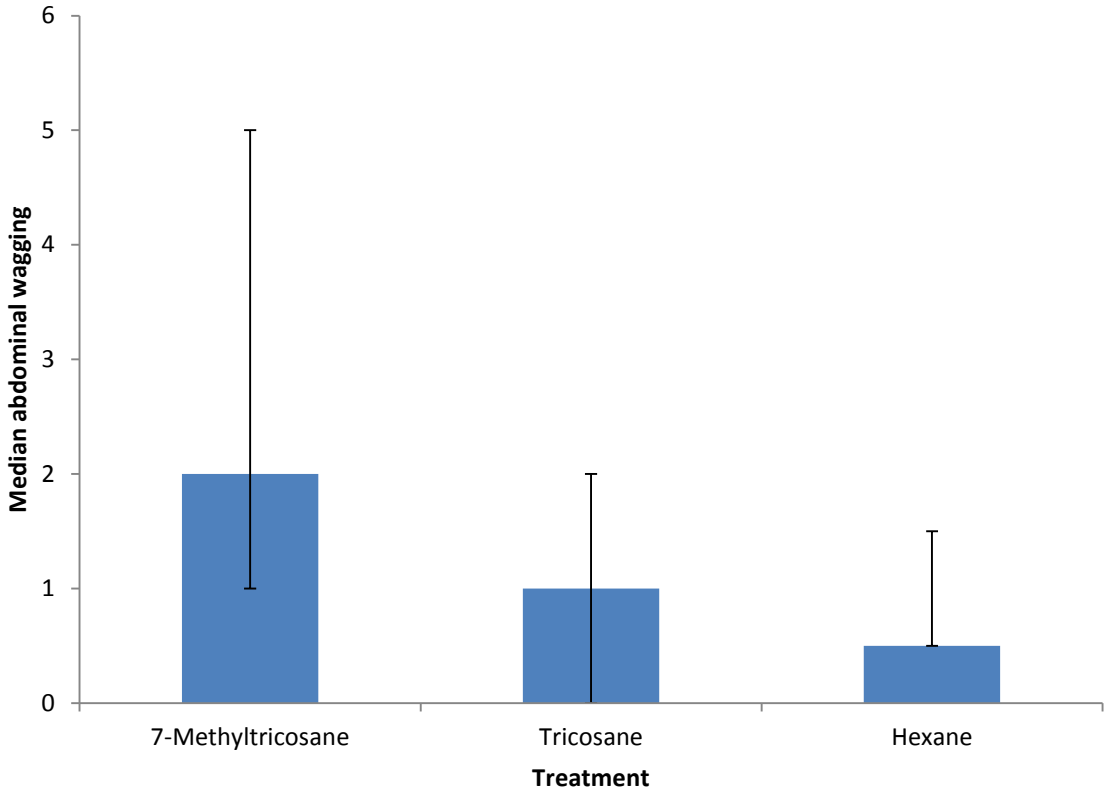

Figure 5.15 The median (± SE of the median) number of times adult male *F. occidentalis* wagged the abdomen in response to synthetic pheromone (7-methyltricosane) and two controls (tricosane and a solvent-only blank) for 3 min after contact with it on glass beads. (Kruskal-Wallis, H= 19.38, *df*=2, *P*<0.001) n=12 trials.

5.4 Discussion

The analysis of exposed discs revealed that a large amount of 7-methyltricosane was found on male-exposed discs while discs exposed to females did not show any presence of this compound. The amount present was higher than the known compounds, neryl (*S*)-2-methylbutanoate or (*R*)-lavandulyl acetate. The compound has an estimated

boiling point of 510 ºC (pers. Comm., D. Hall) which shows it has much lower volatility compared to neryl (*S*)-2-methylbutanoate which has 340 ºC (pers. Comm., D. Hall), it is therefore likely to be placed through physical contact with the filter paper disc rather than being absorbed from the air. However, some cuticular hydrocarbons similar to 7-methyltricosane have been implicated to act as attractant for insects. For example, (Z)-9-tricosene acts as a sex attractant pheromone for female housefly, *Musca domestica* L. (Diptera: Muscidae) ((Carlson *et al*., 1971). This compound attracts male housefly and it has been developed and used commercially for control of housefly (Butler & Mullens, 2010). 7-methyltricosane has boiling point of 411.8 ºC at 760 mmHg, density of 0.796 g/cm^3, refractive index of 1.445 and flash point of 157.9 ºC while (Z)-9-tricosene has boiling point of 300 ºC at 760 mmHg, density of 0.806 g/cm^3, refractive index of 1.445 – 1.454 and flash point of 157.9 ºC. Therefore, further experiments will be needed to ascertain the volatile range of 7-methyltricosane, most especially in the field. This could be done alone and in conjunction with other male-produced compounds of *F. occidentalis.*

This high amount produced suggests that the compound must have a valuable function for male *F. occidentalis* because of the metabolic cost involved in producing it. Though, accurate measurement of metabolic cost of producing a pheromone is lacking. Johansson and Jones (2007) reported that trade-offs exist between pheromone production and fitness-related factors in a wide range of insect species. The metabolic cost of producing and responding to pheromones is likely to determine when an individual should signal and which sex produces the signal (Johansson & Jones, 2007). Svensson (1996) reported that the majority of moths produce pheromone cheaply but the search cost is high, it was suggested that the female is most likely to produce such compounds but conversely when pheromone production is costly; males are likely to produce the pheromone (Johansson & Jones, 2007). This was based on the premise that females invest heavily in reproduction

success and least effort in mate attraction (Kokko & Monaghan, 2001). With the cost involved for males to produce such compounds, it is most likely to be involved in mate choice. Species recognition, mate recognition and mate assessment are involved in the mate choice process (Johansson & Jones, 2007).

Distance response bioassays showed that 7-methyltricosane does not attract from a distance and thrips only responded to this compound after contact. But after contact with the compound, both male and female *F. occidentalis* stayed and spent more time with the glass bead dummy injected with this compound. When adult female *F. occidentalis* is in contact with the compound, it responds for mating. The observed behaviour of raising abdomen by females shows rejection of mating attempts by males as described previously by Terry and Schneider (1993). The adult females used in this bioassay are mixed age, most of them are mated females, so is not surprising to see rejection behaviour. Furthermore, it has been reported in *Drosophila* species that transferred compounds from male to females are deposited on the female anogenital cuticle and this inhibits remating by other males (Yew *et al.*, 2011). The transfer of 7-methyltricosane to the artificial substrate must be of high importance comparing the amount found on the glass fibre disc during the exposure period of 5 h (175 pg male^{-1}) to the amount found on the male *F. occidentalis* cuticle (198 pg male^{-1}). It is possible that there is a transfer of this compound from male *F. occidentalis* to the adult female during mating thereby rejecting all attempts from other males subsequently. There is a strong indication that most of the females used are mated females, however, it is important to verify this claim by doing experiments with virgin females to have a valid conclusion. Terry & Dyreson (1996) reported that when females mate as a virgin, it takes days or weeks after before they can readily accept a mate again.

Males responded weakly by wagging their abdomen sideways which represents behaviour associated with male-male competitions (Terry & Schneider, 1993). This weak

response may partly be explained by the nature of male *F. occidentalis*, they are known for their aggregation, so it may be difficult to truly observe single thrips acting as they would in a natural aggregation. This further suggests that the compound may be used during aggressive interactions to determine dominant or subordinate males based on their level of pheromone production, though, there is no direct evidence. In some flies the level of production of pheromone by males determines the mating success (Jones & Hamilton, 1998; Shelly & Kennelly, 2003). Also, it may act to convey the male dominance and quality in female mate choice as observed in a cockroach, *Nauphoeta cinerea* (Moore & Moore, 1999). Since 7-methyltricosane is a less volatile compound than the aggregation pheromone, neryl (*S*)-2-methylbutanoate, it is quite likely that adult males used it to scent-mark an area which they defend vigorously against any rival male (see chapter 7).

These bioassays show that 7-methyltricosane is used for recognition of species and sex by *F. occidentalis*. However, to be more confident and make a valid conclusion on its role for species recognition, further experiments will be needed with other thrips species to determine the specificity of the compound. This is to confirm whether is broadly used for species or mate recognition. Furthermore, this compound has been reported to be an important component of the species recognition pheromone in the beetle, *Adalia bipunctata* (Hemptinne *et al.*, 1998). This is done after contact with the compound; therefore, the compound is a contact pheromone. Many hydrocarbon compounds have been reported as contact pheromones in insects (Ginzel & Hanks, 2003; Mutis *et al.*, 2009; Blomquist & Bagneres, 2010; Ginzel, 2010), but this is the first evidence of a contact pheromone in Thysanoptera.

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Chapter 6

Behavioural responses to natural pheromones on a natural substrate

6.1 Introduction

As highlighted in chapters 4 and 5, *F. occidentalis* has been shown to respond to pheromones released on filter paper discs. These behavioural responses under laboratory conditions provided useful information on the pheromonal communication of this important worldwide insect pest of major agricultural crops.

The laboratory bioassays have shown that female *F. occidentalis* responds to male-produced compounds on artificial substrates (Chapters 4 and 5). The filter paper and glass fibre discs used have been widely used for laboratory bioassay (Koschier, 2006; Webster *et al.*, 2006; Dublon, 2009). However, there is a need to determine whether adult male *F. occidentalis* applied the compounds on the artificial substrate through sternal rubbing, perhaps from sternal glands, or whether the compounds were absorbed from the air by the filter paper discs. Comparing responses observed with a filter paper disc and natural substrate may give a better understanding of what occurs in the field. Therefore, using a natural substrate may help explain this phenomenon. As discussed in chapter 3, laboratory bioassays should be a mirror of what is expected in the natural field environment, a way to achieve this was to use natural plant materials in the laboratory bioassays.

Many studies have used various natural plant materials, whole plant, leaf disc, wood, twig and log for their bioassays (see Hare, 1998). The material to be used is strongly influenced by the kind of behaviour to be measured and the natural host of the insect involved, for example, termite behaviour will be properly measured using woods and logs. There must be a careful consideration of the materials to be used, factors like age, damage, disease and even water content of the material should be taken into account. All these factors are important in order to minimise variation and to have a consistent behavioural response.

A whole plant may be used but more conveniently, a leaf disc cut from a whole plant may provide a better way to measure the behavioural responses due to homogeneity of such leaf discs. However, the leaf disc should be excised in a way that leaf physiology is not unacceptably damaged, introducing a bias in the result of the bioassay. Leaf discs are also more suitable because of their uniformity in size and shape compared to a whole plant. They can be arranged with ease on any chosen arena, such as Petri dishes (Hare, 1998).

6.1.1 Experimental aims

This chapter attempts to detect whether the compounds are applied or absorbed from the air by examining the behavioural differences of female *F. occidentalis* under laboratory conditions using chrysanthemum leaf as a natural substrate compared to an artificial substrate, filter paper disc. This chapter also attempts to reproduce the behavioural response to natural pheromone under a field condition by using a living substrate. This is to gain more insight into the effect of substrate on behavioural response of adult female *F. occidentalis* to pheromone.

6.2 Materials and methods

The application method of natural pheromone produced by the male *F. occidentalis* was investigated using a natural plant substrate. This was assayed in the same way as in the filter paper disc bioassay used in the development of pheromone bioassays in chapter 3 except that a chrysanthemum leaf disc was used as the substrate. Chrysanthemum leaf was used mainly because thrips were reared on this plant and they can be used for longer exposures before drying up compared to flower petals. Accessibility and convenience were other factors considered for choosing the substrate. This assay attempts to reproduce the occurrence in the field and to detect any obvious biological response of female *F. occidentalis* to natural pheromone in a natural environment.

6.2.1 Obtaining leaf discs

A chrysanthemum leaf disc (20 mm diam.) was cut from a fresh potted chrysanthemum plant using a cork borer. The leaf discs were exposed to 15 male *F. occidentalis* for 5 hours as described in section 3.2.2. The exposure of the leaf discs to thrips was carried out in the early hours of the day from 08:30 – 14:30 h which is the period already established that thrips showed good responses to exposed discs in the laboratory (see Chapter 3).

6.2.2 Leaf disc choice bioassay

The leaf disc bioassay was similar to the filter paper disc bioassay (see 3.2.3) except for the type of substrate used. Leaf discs (male-exposed and control) were placed on the two positions marked on the Petri dish using forceps cleaned with hexane (n-hexane, pesticide residue analysis grade (1526764), VWR International Limited, Poole, UK) as described in 3.2.3.

The bioassay was carried out as descried in 3.2.3. The experiment consisted of 12 replicates initially, however due to the marginal *P* value obtained, it was necessary to do a few more replicates to have a more valid conclusion thereby having 15 replicates in total. The bioassay room was maintained at a constant temperature as described in 3.2.2.

6.2.3 Leaf disc no-choice bioassay

To detect behavioural responses (walking and flits) of female *F. occidentalis* both in the presence and absence of pheromone using a natural substrate, a no-choice test was used. Leaf discs were exposed to 15 male *F. occidentalis* to obtain male-exposed leaf discs as described in 3.2.2. Thereafter, a set of four 40 mm Petri dishes and corresponding lids (Anumbra, Fisher Scientic, UK) were prepared as described in 2.4. The bioassay was carried out as descried in 4.2.2. The experiment consisted of 12 replicates in total. The bioassay room was maintained at a constant temperature as described in 3.2.2.

6.2.4 No-choice bioassays: observations

The number of thrips moving within the arena was recorded as a walking response while flight and landing was measured as a flit. These were recorded over a 30 minute period, every 3 min for walking and continuously for flits. A walking response was recorded when a thrips moved within the arena over the glass surface or leaf disc. Flits were recorded as an event when a thrips leaves any part of the arena with a flight/take off and landing.

6.2.5 No-choice bioassay: statistical analysis

The walking and flit responses of female western flower thrips were recorded as described in 4.2.7. The walking response data was analysed using General Linear Model ANOVA. However, flits data were not normally distributed due to the presence of extreme

values. Log transformation was then applied to the flit data before using General Linear Model ANOVA. The data were analysed with Minitab 16.

6.2.6 Choice bioassay: observations

As described in 3.2.1, the arena measurement method was used throughout the choice bioassays. The Petri dish was divided into two equal halves using the treatment and control filter discs as the basis of division. The number of adult female thrips on each half of the Petri dish was counted and recorded. Records were taken every 3 min for a total of 30 min giving 10 recordings per bioassay. However, other behavioural activities were observed and noted for any variations which may assist in the interpretation of the results.

6.2.7 Choice bioassay: statistical analysis

Data collected were averaged for each bioassay and processed to obtain a Response Index (RI) as described in 3.2.5. The RI was analysed using General Linear Model ANOVA (GLM) and all data were tested for normality as described in 2.7. The data were analysed with Minitab 16.

6.3 Results

6.3.1 Response of adult female *F. occidentalis* to natural pheromone in a choice bioassay

The response of adult female *F. occidentalis* was measured when presented with a male-exposed leaf disc and a control (non-exposed) leaf disc in a choice bioassay. There was a significant preference by adult female *F. occidentalis* for male-exposed leaf discs than the non-exposed control leaf disc ($t_{(14)} = 2.83$, $P = 0.013$). The mean response index (± SEM) was 0.04 ± 0.014.

6.3.2 Response of adult female *F. occidentalis* to natural pheromone in a no-choice bioassay

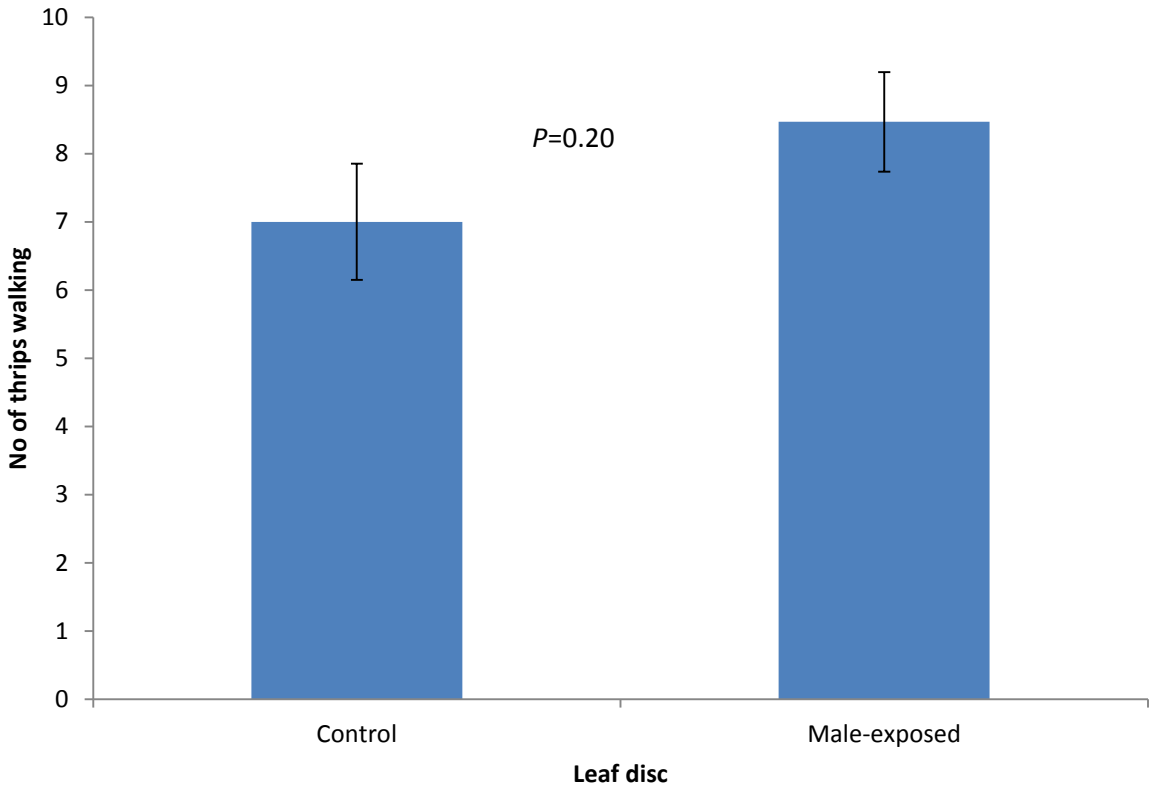

Figure 6.1 The mean (± SE) number of thrips walking on the chrysanthemum leaf disc during the bioassay period of 30 min. There was no significant difference in the walking activity of adult female thrips on the leaf discs. (ANOVA, $F_{1,29} = 1.71$, $P = 0.20$). n= 12 trials.

As shown in Figure 6.1, there was no significant difference in walking response of adult female *F. occidentalis* when presented with the male-exposed leaf discs and non-exposed leaf discs (ANOVA, $F_{1,29} = 1.71$, $P = 0.201$). Similarly, the number of flits recorded by adult female *F. occidentalis* with male-exposed leaf discs was not significantly different from the non-exposed leaf discs (ANOVA, $F_{1,29} = 3.09$, $P = 0.090$) (Figure 6.2). However, in both cases the trend was in the predicted direction.

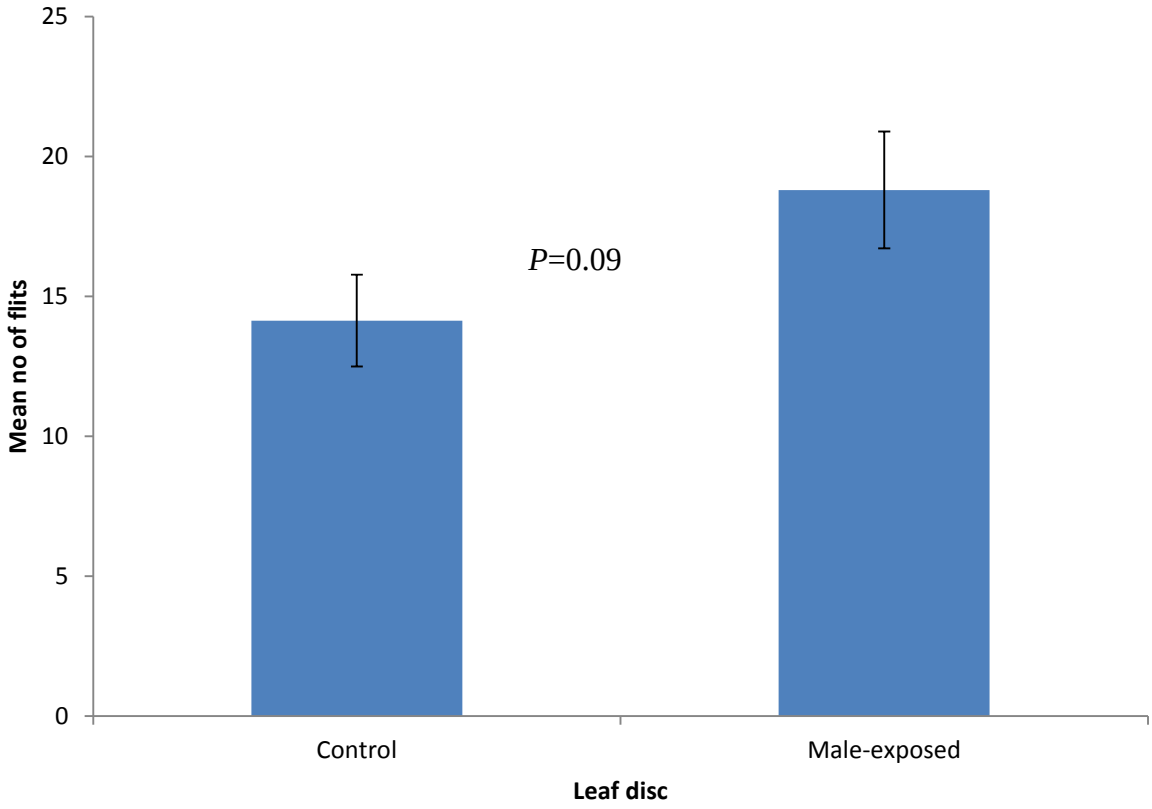

Figure 6.2 The mean (± SE) number of flits on the chrysanthemum leaf disc during the bioassay period of 30 min. There was no significant difference on the flit activity of adult female thrips on the leaf discs. (ANOVA, $F_{1,29}$ = 3.09, P = 0.09). n=12 trials.

6.4 Discussion

Adult female *F. occidentalis* responded significantly to male-exposed discs when natural pheromone was on artificial substrates (Chapters 4 and 5). Their activity level increased, walking and flitting more in the presence of the male-exposed discs. Similarly, adult female *F. occidentalis* preferred the leaf discs exposed to adult male *F. occidentalis*, though this was achieved when more replication was done but it thus suggests that female *F. occidentalis* were able to detect and respond positively to compounds on the exposed

leaf discs. However, increased activity level recorded with artificial substrate could not be demonstrated using the chrysanthemum leaf disc, though adult female *F. occidentalis* walk and flit more with the male-exposed leaf discs but not significantly more than with the non-exposed leaf discs. The possible explanation for this kind of result may be the compound present on the leaf disc. Neryl (*S*)-2-methylbutanoate is an attractant (Hamilton *et al.*, 2005) and increases the activity level of female *F. occidentalis* (see Chapter 4). Being a volatile compound readily absorbed by filter paper discs, it is less likely to be on the leaf disc because of the waxy surface of the leaf which may not absorb the compound. (*R*)-lavandulyl acetate is probably a short range attractant (see chapter 4) and reduces the walking and flitting activities of female *F. occidentalis*. The possibility of this compound on the leaf disc is less likely as well because is a volatile compound. However, 7-methyltricosane may be the compound present on the leaf disc which female *F. occidentalis* were responding to by choosing the male-exposed leaf disc more than non-exposed. The compound is not volatile and may be on the leaf through ventral abdominal contact. Male *F. occidentalis* were observed to be in contact with the leaf discs during the exposure experiment and this may account for the female response.

The colour of natural substrate used may have influenced the activity level of the thrips. Behavioural activities of *F. occidentalis* are influenced by colour (Matteson & Terry, 1992; Blumthal *et al.*, 2005; Mainali & Lim, 2011; Broughton & Harrison, 2012). They are attracted by brighter coloured objects; however, the chrysanthemum leaf substrate is green in colour which is not an attractive colour to *F. occidentalis*, which may limit their patrolling and other activities necessary for the production of certain pheromone. In the same vein, *F. occidentalis* is a flower dwelling insect, this may further contribute to low level of activities on the leaf disc and they may be engaged in other tasks like feeding thereby not producing pheromone.

One of the important morphological characteristics of a chrysanthemum plant is the leaf hairness (De Jager *et al.*, 1995), this may also inhibit the walking and patrolling activities of *F. occidentalis* thereby reducing or totally affecting pheromone production. This may possibly account for the low number of *F. occidentalis* walking observed, because of the resistance to movement on the substrate.

The nature of the chrysanthemum leaf may also contribute to the observed result. The results suggest that one of the compounds; 7-methyltricosane may be applied on the leaf while the other two compounds may not. If the leaf is unable to absorb those two male-produced compounds, it simply means that adult female *F. occidentalis* will respond to the leaf disc in a similar way to non-exposed leaf disc. While the response using a choice bioassay was significantly different between exposed and non-exposed this was not distinguishable with a no-choice bioassay. And this clearly showed that certain compounds may not be present or at different levels on the natural substrate compared with artificial substrate thereby accounting for the differential response by adult female *F. occidentalis* or simply due to the morphological characteristics of a chrysanthemum leaf.

Though, no significant response was recorded with the chrysanthemum leaf disc no-choice bioassay, this need to be tested further in order to make a valid conclusion on the compound present on the leaf disc and justify the suitability of such natural substrates for the bioassay. However, the choice bioassay clearly showed that an active compound was on the leaf disc which may be non-volatile and is likely to be 7-methyltricosane (see Chapter 5) (Olaniran *et al.*, 2013).

More experiments could be carried out using other natural substrates like yellow or white flowers which attract *F. occidentalis* (Matteson & Terry, 1992) to detect if the hairiness of the leaf inhibits the walking activity of the thrips. While a chrysanthemum leaf

disc is not the ideal natural substrate for this bioassay, the results show that pheromones are transferred to natural plant substrates.

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Chapter 7

Fighting behaviour of adult males[1]

7.1 Introduction

Evolutionary game theory (Maynard-Smith, 1982) has been utilised to provide information on the aggressive behaviour of animals. This aggressive behaviour over valuable resources is a naturally occurring phenomenon and has been widely reported (reviewed by Andersson, 1994; Kemp & Wicklund, 2001). The outcome of this behaviour has significant consequences for resources gained or lost, energetic cost of contest and the ultimate risk of being injured (Briffa & Sneddon, 2007). The valuable resources contested by insects include mates, food, oviposition sites and territories (Moore *et al.*, 2008). However, the insect mating system depends on availability of females (sex with limited availability), which invariably leads to potential monopolisation of females by males (Kroiss *et al.*, 2010). Males can achieve this monopolisation of females by guarding the females or resources important to females which include food or oviposition sites.

This behaviour leads to competition among males because of value attached to the resources. Males assess each other using their fighting ability (resources holding power, RHP) (Parker, 1974) which is typically influenced by body size, weapon size or enlarged structures modified for fighting, experience, age and other factors. To attract females, males can also use visual, acoustic and chemical cues as well as physical fighting (Shelly

[1] The results have been published (Olaniran & Kirk, 2012)

& Whittier, 1997; Kroiss *et al.*, 2010). Body size has been shown to strongly influence the results of competitive fights between males in insects (Crespi, 1986; Terry & Dyreson, 1996; Elias *et al.*, 2008; Briffa, 2008; Reaney *et al.*, 2011). Larger competing males usually win the contest and hence the choice of female for such large males (see Choe & Crespi, 1997). However, recent fighting experience has also been shown to have a strong effect on the fighting ability of insects. The changes in an individual's perception of its status as a winner or loser from previous contests can greatly affect the fighting ability or competitiveness with a new rival/opponent (Hsu *et al.*, 2009). The re-assessment of the previous contests has the potential to supersede the body size effect on the new fights (Kasumovic *et al.*, 2009). Reaney *et al*. (2011) concluded that previous fighting experience only affects contest outcome during non-physical encounters, but did not alter the effect of body size on behaviour and outcome if a male could directly assess the fighting ability of his opponents. Similarly, size of fighting weapon can affect the fighting ability of an individual as is found in beetle horns and mandibles (reviewed in Emlen, 2008) and enlarged forelegs in *Elaphrothrips tuberculatus* (Crespi, 1986).

The assessment strategy is highly important in the contest over limited but valuable resources. It gives opportunity to the competing males to process different forms of information to weigh the available options and economics of engaging in the resource competition (Keil & Watson, 2010). During any interaction or contest, three sources of information are available: self, opponent and resources. Self-assessment estimates the RHP which includes physical ability, residual mating value, energy level and previous fighting experience. An opponent will estimate self RHP relative to his rival to determine the level of commitment needed to put into the contest. The end result of female receptivity and quality of potential mates will offer the estimate of resources. However, estimation of the

resource factor should also consider using the costs of obtaining alternative potential mates of similar value (Arnott & Elwood, 2008).

Many male insects competing for resources may compensate for small body size by adopting an alternative mating approach. Mating success as a result of body size has been reported in many insects, large males have advantage in competing for mates and resources (Thornhill & Alcock, 1983; Crespi, 1988; Wang & Zeng, 2004; Ray *et al.*, 2009; Reaney *et al.*, 2011). However, smaller males avoid aggressive competition with larger males by searching for resources that are left unguarded by any large males or intercepting females prior to being discovered by larger males by moving around the resources location.

In some insects, though, males that engage in fights are of similar size, while those of different class size tend not to engage in fights after assessing the costs and benefits of such fights. The males with similar size engage in fighting probably after assessing the RHP and conclude that benefits from fighting far outweigh the costs attached to such competition.

In Thysanoptera, male aggregations are common (Kirk, 1985; Terry & Gardner, 1990) and presumably associated with mating or the search for food. However, these male aggregations appear to be primarily for mate location, feeding and oviposition (Kirk, 1985; Terry & Dyreson, 1996). During mating aggregations, no aggressive interactions among males or females were observed in *Thrips fuscipennis* Haliday, *Thrips major* Uzel (Kirk, 1985) or *F. schultzei* (Milne, 1997), but were observed among males of *F. occidentalis* (Terry & Dyreson, 1996) and *F. intonsa* (Kirk, 1996). Contacts between *F. occidentalis* males consisted of either a brief interaction or a short single bout of abdominal flicking before parting. The fighting between a pair of males included longer contact with mutual abdominal flicking before parting; however, the escalated fights which included grabbing

and flipping were rare. A detailed description of these behaviours has been provided by Terry & Dyreson (1996).

Within aggregations, the fights between male *F. occidentalis* possibly occurred to clear the landing area on the flower corolla lobes where females mate when they land (Terry & Dyreson, 1996). The recent identification of a non-volatile compound, a contact pheromone in *F. occidentalis* (Olaniran *et al.*, 2013) suggests that the pheromone may be involved in the aggressive male-male interactions. It was observed that in the presence of this pheromone adult male *F. occidentalis* wagged their abdomen sideways which is a behaviour described previously to be associated with fighting in *F. occidentalis* (Terry & Gardner, 1990). When females enter the aggregation, they generally mate with the first male encountered and then refuse advances from most other males (Terry & Dyreson, 1996). It is not known whether mate choice and selection within an aggregation is by visual or chemical cues. Pheromones are used by *F. schultzei* to attract females (Milne, 1997), *F. occidentalis* to attract both males and females (Hamilton *et al.*, 2005) and by *F. intonsa* (Zhu *et al.*, 2012). *Thrips* species males detect a female within a few millimetres (Kirk, 1985). It was further suggested by Lewis (1973) that sexes find each other by odours with the use of sense cones on the antennae. In sexual selection of many insects, pheromones are an important cue and both long-range and short-range chemical signals have been found to attract mates (Svennsson, 1996; Phelan, 1997).

Behaviour within aggregations is affected by the density of male *F. occidentalis*. At very high densities, escalated fights were not observed (Terry, 1995). Kirk (1985) reported that density changed the behaviour of male *Thrips fuscipennis* and *Thrips major*.

7.1.1 Experimental aim

This study measures the effect of density on the behaviour of male *F. occidentalis* within aggregations in a laboratory environment. This was carried out to understand the aggressive interactions and territorial defence in male *F. occidentalis*.

7.2 Materials and Methods

7.2.1 Arena technique

A pollen-feeding technique as described by Kirk (1987) was adapted for this study. A piece of dental wax (30 mm long, 23 mm wide, and 1.5 mm thick) had a 9 mm diameter disc removed from the centre with a cork borer (C70-410 No. 5 Orme Scientific, Manchester, UK). This hole formed the arena for the observation of thrips behaviour. The calculated area of the arena was 63.6 mm^2. The wax was pressed onto the middle of a microscope slide (76 mm long, 26 mm wide) which formed the floor of the arena. They were sealed together by a gentle warming of the slide in a flame, in order to soften the wax; they were then pressed together again to give a secure seal. A glass cover slip (16 mm diameter, 0.16-0.19 mm thick) was pressed down gently into the wax to form the transparent roof of the arena.

7.2.2 Bioassay

Mixed-age adult males were collected with a small aspirator in required quantities and briefly anaesthetised with a gentle 10 s stream of carbon dioxide (British Oxygen Company, UK). They were carefully transferred into the arena and immediately covered with a glass cover slip. This arena was placed on a rectangular shelf, 600 mm long and 200 mm wide, to allow for consistency with established bioassay procedures, in the bioassay room with a constant temperature of 25±2ºC and illuminated from above with four,

fluorescent tubes (F65W/35 General Electric, Hungary), providing approximately 1000 lux of light.

7.2.3 Video recording

A mono CCD video camera (Sanyo WCB-3385P, Sanyo, UK) with a 12 mm *f*:1.4 lens (H1214 FICS-3, Computar, Japan) was placed above the arena at a height of 15 cm that enabled it to view the whole arena. This was connected to a video recorder (CTR-3024, Computar, UK) and each experiment was recorded for a duration of 10 min. Data were obtained either directly from the video and/or reconciled with the manually recorded data.

7.2.4 Observations

The experiment was carried out between 09:00 and 10:30 h for consistency over several days. The number of interactions, frequency and duration of the fights were noted during the 10 minutes of observation. An "interaction" was defined as a 0-2 s brief contact and no fighting before separation. A "fight" was defined as a prolonged interaction with abdominal flicking, grabbing and/or flipping. To obtain the rate of interactions, the number of interactions obtained for each density was doubled since it involves two thrips and this was divided by the number of thrips within each density to obtain the number of interactions per thrips. This was also applied to calculate the rate of fights. This was utilised to describe the male – male interactions indicating involvement of two thrips. In calculating the time spent fighting, time of fights for all the thrips were averaged for the 10 min period.

7.2.5 Statistical analysis

All data were analysed by two-way ANOVA and Tukey's test was used for multiple comparisons. Statistical analysis was carried out with Minitab 16 (Minitab Inc., Pennsylvania, USA).

7.3 Results

Thrips were placed in a small artificial arena that could be filmed easily and the behaviour of thrips was observed at densities from 4–16 adult males. It was expected that behaviour would change in relation to density. To describe the experiments, the number of thrips in the arena was categorized into three densities; low density (4-6 thrips), intermediate density (8-10 thrips) and high density (12-16 thrips).

7.3.1 Rate of interaction

The rate at which male thrips made brief contacts without fighting increased linearly as the number of thrips within the arena increased (Figure 7.1). As the density increased, there were more interactions because there were more thrips in the arena and the chance of one thrips encountering another increased. A secondary effect however, was that due to the mobility of the thrips they tended to bump into each other more and so were more active throughout the experiment. This was expected since greater disturbance causes more frequent encounters within the arena. The male-male interaction rate was about four times greater at high density than at low density. The interaction rate was significantly different between the densities (ANOVA, $F_{6,30}$ =27.59, P<0.001) (Figure 7.1), showing the expected trend of a positive linear relationship between the rate of interaction and the number of thrips.

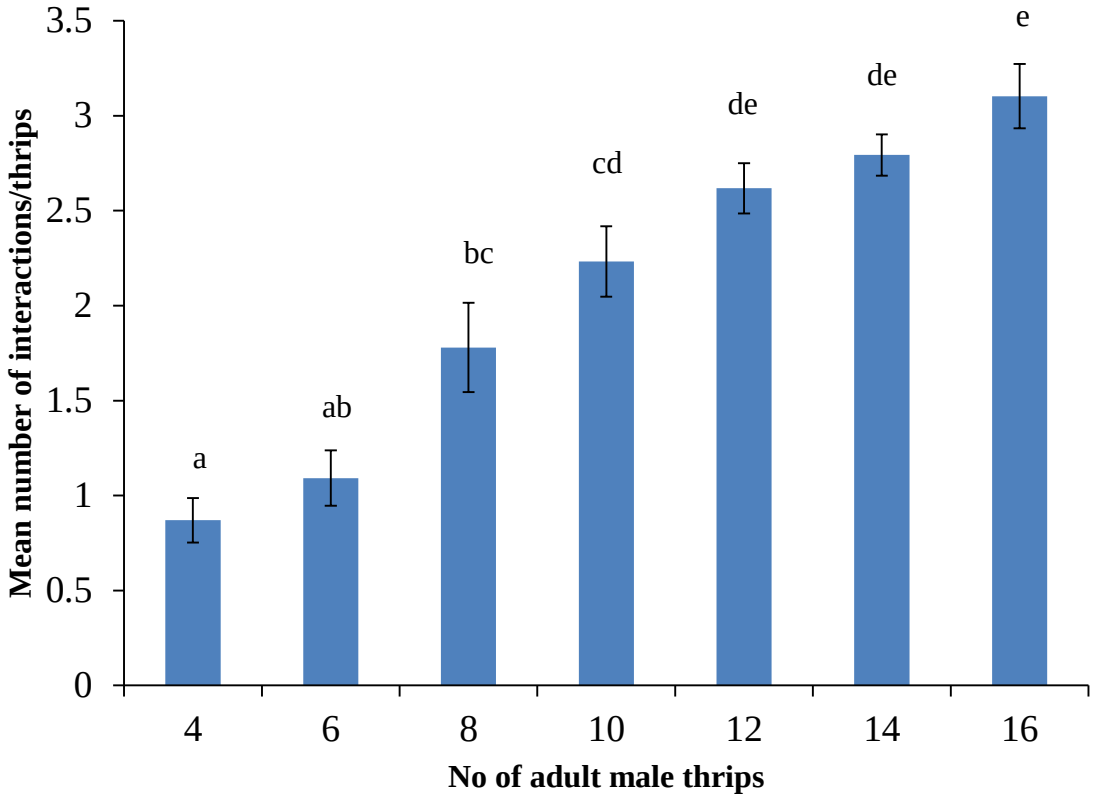

Figure 7.1 The rate of interactions for different male densities during a 10 minute observation in an arena of 9 mm diameter. An interaction was defined as a brief contact and no fighting between a pair of males before separation. Means with different letters indicate significance at $P<0.05$ (Tukey), n=6 trials.

7.3.2 Rate of fights

The rate of fights was found to be highest at the intermediate density, with considerably less fighting at low and high densities (Figure 7.2). The number of fights recorded by an individual within the arena was significantly different across the densities (ANOVA, $F_{6,30}$ =26.43, $P<0.001$). The number of fights observed at intermediate density was about twice that at low density and three times greater than that at high density.

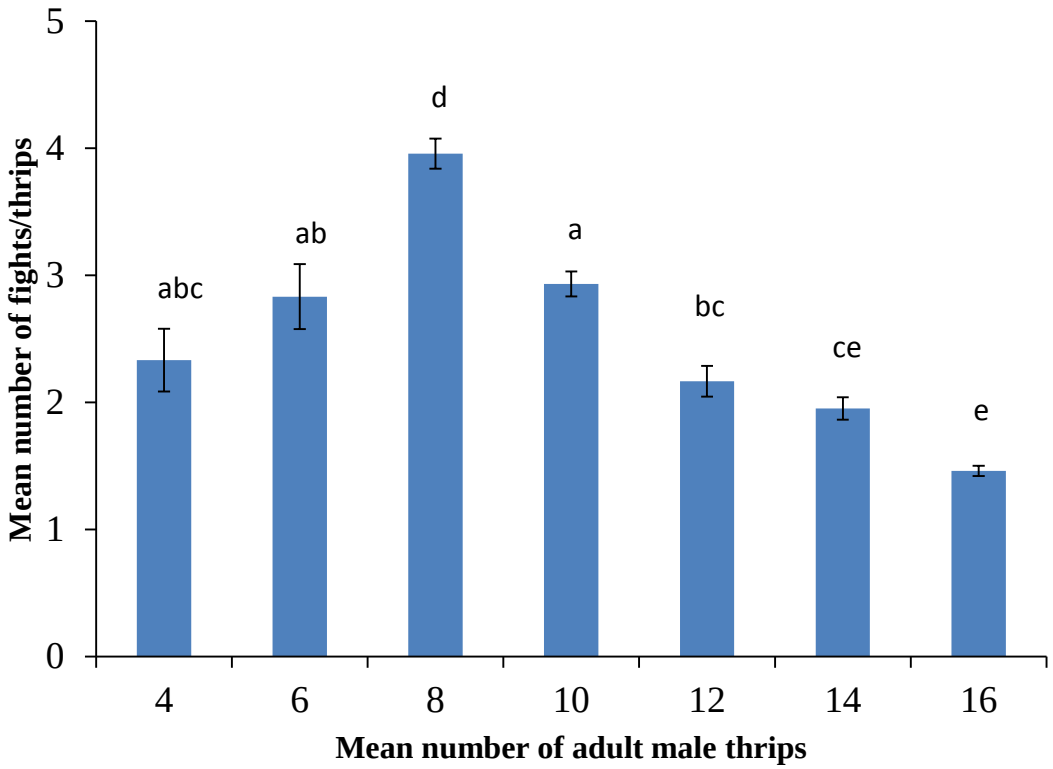

Figure 7.2 The rate of fights per thrips for different male densities during a 10 minute observation in an arena of 9 mm diameter. A fight was defined as a prolonged contact with abdominal flicking, grabbing and/or flipping between a pair of males before separation. Means with different letters indicate significance at $P<0.05$ (Tukey), n=6 trials.

7.3.3 Time spent fighting

The percentage of time spent fighting was significantly different for all the densities (ANOVA, $F_{6,30}=30.29$, $P<0.001$) (Figure 7.3). The longest time was recorded at intermediate density, while the high density had the shortest total time of fights. The mean duration of a fight was also significantly different across the densities (ANOVA, $F_{6,480}=52.21$, $P<0.0001$) (Figure 7.4); the intermediate density had the longest fights which were about three times longer than at the low density. The longest mean fight duration during

the experiment was at a density of 10 thrips (15.9 thrips^{-1} cm^{-2}). At the high density, the mean fight duration was twice the duration at the low density.

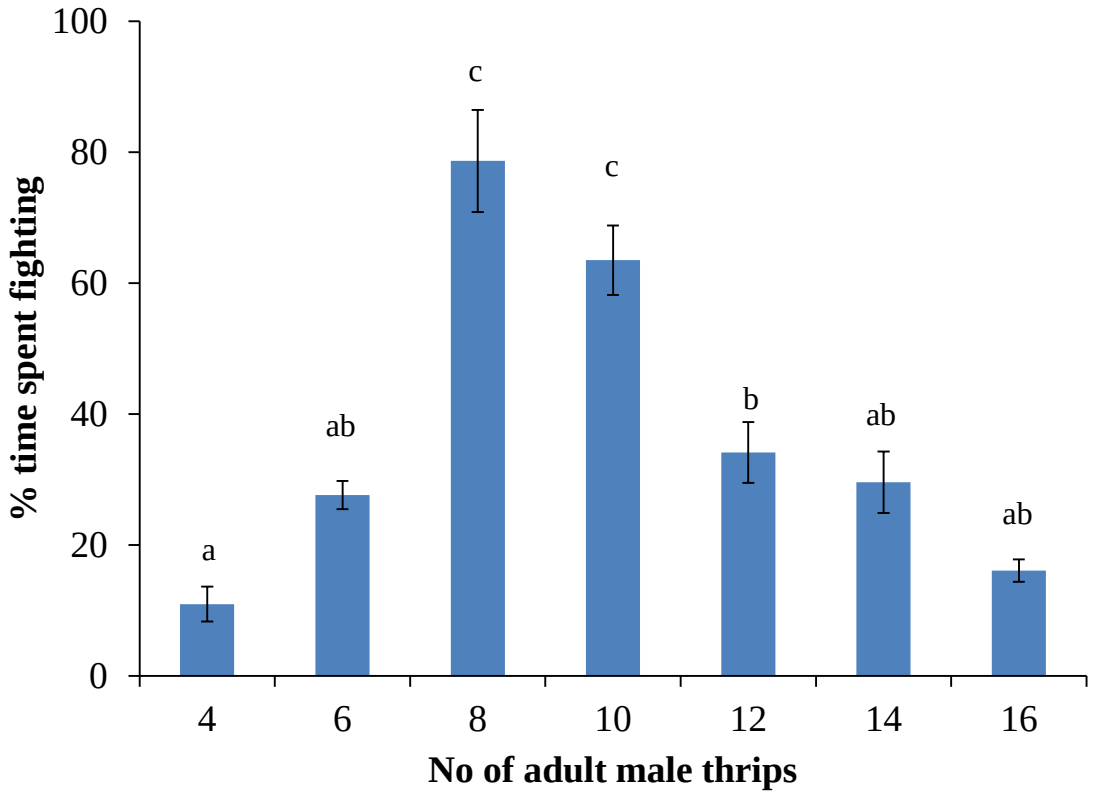

Figure 7.3 The percentage time spent fighting by thrips for different male densities during a 10 minute observation in an arena of 9 mm diameter. Means with different letters indicate significance at $P<0.05$ (Tukey), n=6 trials.

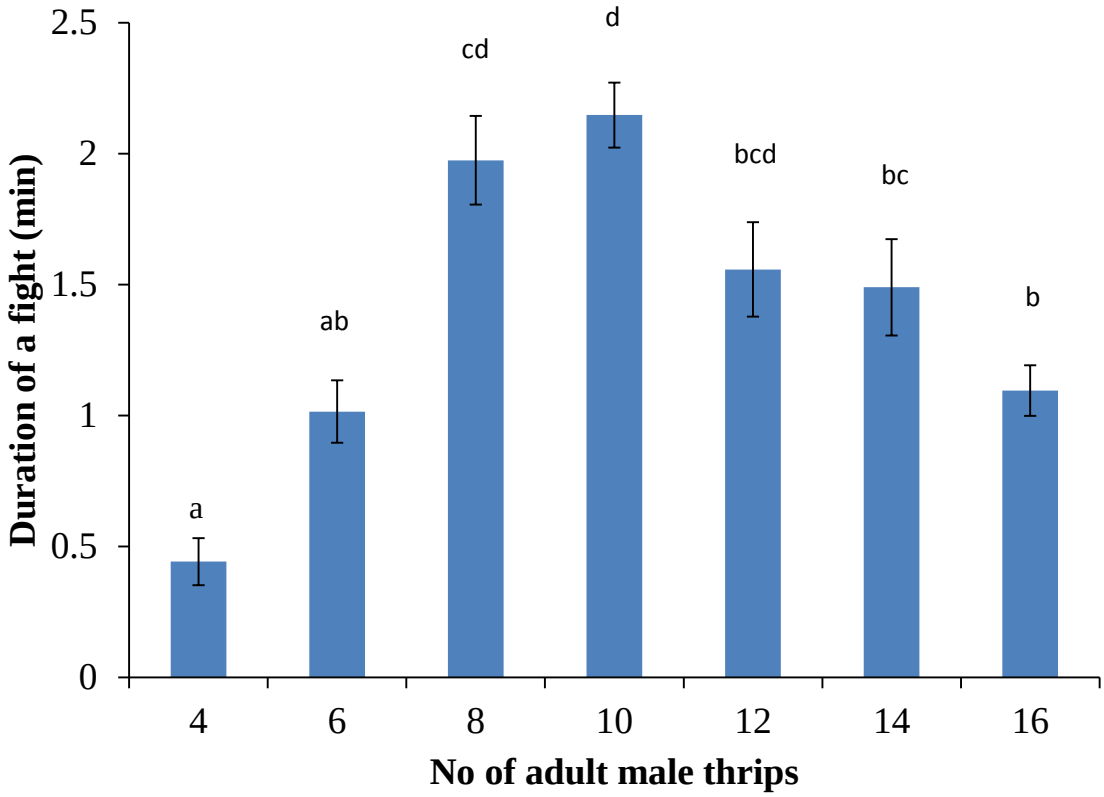

Figure 7.4 Duration of a fight for different male densities during a 10 minute observation in arena of 9 mm diameter. Means with different letters indicate significance at $P<0.05$ (Tukey), n fights=28, 51, 95, 88, 78, 82, and 70 for n trials= 4, 6, 8, 10, 12, 14 and 16 thrips respectively.

7.4 Discussion

The male fighting behaviour was studied using an artificial arena and this made it easy to record and identify the various forms of behaviour associated with fighting that are exhibited by male *F. occidentalis*. Feeding and mating, which are activities involved in aggregations were not studied as female and food sources were not present. Mating aggregation on flowers is similar to that on artificial substrates (Matteson & Terry, 1992). Within aggregations of male *F. occidentalis*, different forms of behaviour were observed,

ranging from brief contact without fighting after meeting to repeated bouts of abdominal flicking, grabbing and flipping.

The rate of interaction increased linearly with density as would be expected if thrips behaved like randomly moving particles. There was no evidence that individual behaviour for interactions was affected by density. However, the relationship between the density and rate at which fights occur did not follow the same pattern as interactions. Male *F. occidentalis* fight less at low and high densities while more fights occurred at intermediate density. At low densities, there may be no advantage in fighting, probably because there is enough space available for each thrips to interact freely. At intermediate density, fighting may be of benefit because it is possible to secure a site for mating without much cost. However, fights at high density seem to be a disadvantage to the thrips, possibly because the space available was limited and extended fighting would be very costly.

Terry & Dyreson (1996) suggested that fights occur to obtain a more strategic position for sighting and intercepting females landing within the aggregation. It is known that when female *F. occidentalis* enter aggregations, they mate and reject advances by other males. This poses questions such as what allows some males to mate successfully with females. What are the qualities and attributes that distinguish successful males from rejected males within the aggregation? A further question is do females mate with the winners of male-male fights. In many insect taxa, the winner of a fight or the dominant male mates with the female, however, loser or subordinate males sometimes mate by patrolling nearby. A female preference for males that win a fight has been demonstrated in many taxa (Berglund & Rosenqvist, 2000; Lopez *et al.*, 2002). A number of studies have reported female preference for the odour of dominant males, e.g. rodents (Kruczek, 1997) and crickets (Kortet & Hedrick, 2005).

It is possible that the winner of fight contests in male *F. occidentalis* aggregations produces an odour that females recognise. It has been reported that males produce several compounds during aggregation (Hamilton *et al.*, 2005), and so compounds produced during fighting within an aggregation could be utilized by females for mating. However, the contact pheromone of *F. occidentalis*, 7-methyltricosane (Olaniran *et al.*, 2013), may be the pheromone involved in the fighting behaviour, possibly the compound is placed on an area where females may likely land thereby preventing other males from gaining access to such landing females. This may lead to escalated aggressive behaviour between males. It has also been reported for the sandfly *Lutzomyia longipalpis* that females prefer males that produce more pheromone and/or wing-fan (Jones & Hamilton, 1998).

At intermediate densities fights had the longest duration; it perhaps indicates that there was a benefit to be derived from longer fights such as ensuring the defeat of an opponent or that the amount of pheromone produced was important in the fight contest and eventually for mating success. Males interact over extended periods, so the contact pheromone could be involved as is found with male sandflies (Jones & Hamilton, 1998). It has been estimated that a male *F. occidentalis* produces the aggregation pheromone, neryl (*S*)-2-methylbutanoate, at a rate of 120 pg male^{-1} h^{-1} (Kirk & Hamilton, 2004; Dublon *et al.*, 2008). Furthermore, it has been reported in the Australian field cricket that male dominance status is associated with the expression of male pheromone signals and that these males obtained a greater number of successful mating (Thomas & Simmons, 2009).

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Chapter 8

General discussion

The main aim of this thesis was to examine the biological roles of pheromones of adult *F. occidentalis* in the laboratory with a view to gaining understanding of their practical utilisation and application in the field. However, in order to achieve this, some specific aims were set: (1) to investigate the aggregation and mating behaviour of the *F. occidentalis* and the potential role of pheromones; (2) to understand the role of (*R*)-lavandulyl acetate in the biology and ecology of the *F. occidentalis* and (3) to attempt to identify any other male-produced pheromones of the *F. occidentalis*.

8.1 Research findings

Adult male *F. occidentalis* produced an aggregation pheromone, neryl (*S*)-2-methylbutanoate and another compound, (*R*)-lavandulyl acetate (Hamilton *et al.*, 2005). While the aggregation pheromone is used commercially, the specific role of the other compound remains unknown. This led to this study to understand its biological function and any other interactions it may have with the aggregation pheromone both in the laboratory and field. Previous findings clearly show that the aggregation pheromone attracts both sexes and this has been utilised in the field in conjunction with blue sticky traps (Hamilton *et al.*, 2005). Apart from the attraction role of neryl (*S*)-2-methylbutanoate, there is no other function attributed to this compound. Therefore, in carrying out this research, both compounds were tested to gain more insight into their activities on *F. occidentalis* and to gain more insight into their activities in the laboratory.

To pursue this research, behavioural responses of adult *F. occidentalis* were tested against natural pheromone and synthetic pheromone. The behavioural responses of adult female *F. occidentalis* in the presence of male-exposed discs were not reproduced when single synthetic compounds were applied. It was proposed that either a combination of the two compounds might explain the differences observed or some other compounds were involved. However, before the combination of the two compounds could be tested, there was an identification of another compound in adult male *F. occidentalis*, and this created another line of enquiry. This has now brought the total number of pheromone compounds identified in *F. occidentalis* to four namely; alarm pheromone: decyl acetate and dodecyl acetate, aggregation pheromone: neryl (*S*)-2-methylbutanoate and contact pheromone: 7-methyltricosane. However, there are other three compounds in *F. occidentalis* but not yet bio assayed to establish their pheromonal role. They are (*R*)-lavandulyl acetate, 7-methylpentacosane and 9-methylpentacosane.

8.1.1 The aggregation pheromone, neryl (*S*)-2-methylbutanoate

It has been established that neryl (*S*)-2-methylbutanoate attracts both male and female *F. occidentalis* in the laboratory and field (Kirk & Hamilton, 2004; Hamilton *et al.*, 2005). This is responsible for the increased trap catches recorded in glasshouses and open field cultivations. In the laboratory bioassay, there was a strong response by adult female *F. occidentalis* towards the synthetic pheromone applied on an artificial substrate, the higher response index recorded strongly supports their attractant role in *F. occidentalis* (see chapter 4). Furthermore, when a no-choice bioassay was used, it clearly revealed that this compound is capable of increasing the activity level of mixed-age adult female *F. occidentalis* (see Chapter 4). There was an increase in the walking and flitting activities of adult females and this observed increase could be utilised in the planning of a control strategy for *F. occidentalis*. Their small size, thigmotactic nature and ability to hide in tight

spaces within plants (Lewis, 1997), reduces effective control of thrips through consequent minimal exposure to insecticides (Shipp & Zhang, 1999). This compound could stimulate the thrips to come out of the tight spaces. This could lead to an increase in the rate of contact between *F. occidentalis* and chemical insecticides or natural predators thereby increasing the mortality rate. However, it is possible that the increased trap catches recorded in the past were partly due to their increased activity level, though it may be difficult to separate attraction from the increase in activity, but both may act in parallel to achieve the recorded results. Other allelochemical compounds also increase trap catches in the field (Kirk, 1985; Teulon *et al.*, 1993, 2007). While pheromones are species-specific, other allelochemicals are not. For example, *p*-anisaldehyde (Kirk, 1985; Teulon *et al.*, 1993) and methyl isonicotinate (Davidson *et al.*, 2008; van Tol *et al.*, 2012) cause an increase in trap catches. Van Tol *et al*. (2012) showed that methyl isonicotinate increases the walking and take-off of *F. occidentalis* in the wind tunnel and neryl (*S*)-2-methylbutanoate showed a similar result in this work. Therefore, neryl (*S*)-2-methylbutanoate not only attracts but also increases the walking and flitting activity of adult female *F. occidentalis*. This result was completely different from what was observed with (*R*)-lavandulyl acetate.

8.1.2 (*R*)-lavandulyl acetate

The bioassay of this compound showed some interesting results for understanding their role in the aggregation and mating behaviour of *F. occidentalis.* (*R*)-lavandulyl acetate was presented to both male and female *F. occidentalis*; the behavioural responses observed were different from each other. When female *F. occidentalis* were tested to determine their preference for this compound, the filter paper disc treated with this compound was chosen significantly more than a control filter disc paper, though the response index was lower compared with what was recorded for the aggregation

pheromone but it clearly showed that (*R*)-lavandulyl acetate can be detected within a short range by adult female *F. occidentalis.* Another striking feature was that this detection can be achieved over a wide range of doses, as low as 50 pg.

The activity data further showed adult female *F. occidentalis* walk significantly less in the presence of (*R*)-lavandulyl acetate treated discs compared with thrips in the presence of control filter paper discs and a similar result was recorded with the flit response. However, male *F. occidentalis* walk and flit significantly more with (*R*)-lavandulyl acetate treated discs than control discs. The increase in male thrips activity level could be explained by their struggle to search for mates, if this compound is released by male when mating to calm female, other males may be activated to locate the female. The observed behaviour in the female *F. occidentalis* suggests that they are arrested which led to significantly less walking and flitting. However, this causes female thrips to remain within the observation arena. If food was provided during the experiment, it could be suggested that the calmness was due to their engagement in other activities like feeding but no food was provided. The results suggest that the compound may be a calming pheromone which makes the female thrips receptive to adult male *F. occidentalis* during the mating process.

Terry (1997) suggested that short-range behaviour or chemical cues are important for adult thrips in colonies or aggregations to advertise their sexual readiness. It is likely that this compound is released by adult male *F. occidentalis* only when a receptive female is sighted as the compound is only produced on demand and not stored up by adult male *F. occidentalis* (Dublon *et al.*, 2008). The reduction in thrips catches in the field (Hamilton *et al.*, 2005) further suggests that the compound does not increase the activity level in adult females as happens with aggregation pheromone and is not a long-range compound (Hamilton *et al.*, 2005). In *Pezothrips dianthi* (= *Taeniothrips dianthi*) a lipoid volatile pheromone calmed the female during mating and attracted other male to the mating pair

(Pelikan, 1951), which suggests that (*R*)-lavandulyl acetate may have a similar function in *F. occidentalis.* In *F. occidentalis* (Terry & Schneider, 1993) and *F. schultzei* (Milne, 1997) calm periods were observed during copulation. During the mating process, a female show her receptiveness by 'ducking' or 'squatting' down with her legs and lowering the abdomen (Terry & Schneider, 1993), though this was not noticed during the experiment but will be investigated in future to make a firm conclusion. A receptive female *F. occidentalis* allows male *F. occidentalis* to climb on her back while an unreceptive one raised her abdomen up to dislodge the male (Terry & Schneider, 1993). However, female *F. occidentalis* may be receptive at the beginning but later reject the male before the mating process ends. This shows the complex behaviour involved in the mating process of adult *F. occidentalis.*

8.1.3 The contact pheromone, 7-methyltricosane

The complex behavioural responses are probably correlated with the number of pheromones identified so far in adult male *F. occidentalis*. The major differences observed in the behaviour of adult female *F. occidentalis* to natural pheromone (male-exposed discs) and synthetic compounds led to a search for a possible explanation. The identification of new pheromone compounds was pursued to ascertain their biological role in *F. occidentalis*. While male-exposed discs revealed the presence of 7-methyltricosane, the cuticular extracts of adult male *F. occidentalis* showed the abundance of cuticular hydrocarbons. 7-methyltricosane was the most abundant in adult male *F. occidentalis;* there was a trace in females and none in larvae (Olaniran *et al.*, 2013). This compound was found to be used in species recognition by adult *F. occidentalis.* Both adult male and female recognised a glass bead coated with this compound and responded to it as if it were a male of the same species, but the same response was not observed with *n*-tricosane which is found only in small amounts in the cuticular hydrocarbons of both sexes or with hexane-

control glass beads. The response to 7-methyltricosane was observed after contact with the glass beads which suggests that the compound was not acting at a distance. This was compared with a known attractant, neryl (*S*)-2-methylbutanoate, the aggregation pheromone. Adult male and female *F. occidentalis* moved towards the aggregation pheromone in a distance bioassay, however, this was not the same with 7-methyltricosane which revealed it can only be detected after contact with the compound.

In another experiment, fighting among adult male *F. occidentalis* showed that male density has an effect on fighting behaviour. There is an optimum density at which fighting is most important to adult males while at low and high density, fighting is very low compared to the optimum density. This clearly shows that at the optimum density, there is a benefit which outweighs the cost of fighting. The low fight rate recorded at low and high densities shows the reverse. The contact pheromone may be involved in this cost-benefit ratio. The pheromone is applied on the surface and possibly adult males were able to measure the level of the pheromone. In lekking sandflies, fighting occurs and it has been found the amount of pheromone produced determined the mating success of males (Jones & Hamilton, 1998). The amount of 7- methyltricosane produced with exposed discs and on their cuticle suggests its usefulness in the aggregation and mating behaviour. The metabolic cost that must be involved shows the high importance of the pheromone production. More experiments need to be undertaken to understand more about this compound. Though the mating system of *F. occidentalis* has been described as resource-based (Terry & Dyreson, 1996), there is the possibility a lek is involved. A lek was defined by Bradbury (1981) and this was modified by Bradbury (1985). A lek is defined as where all these criteria are met: (a) no male parental care, (b) males occur on territories which are spatially clustered within a population's accessible range, (c) there is no male-regulated access to resources which influences female visits or mate choice, and (d) females have the

freedom to select a mate in the male territories. However, Höglund and Alatalo (1995) defined a lek much less strictly as an arena where males aggregate and stay until females land. Females come to these places with the sole aim of mating, and do not receive any other resources from mated males.

To qualify as a lek according to Bradbury (1985) all the four criteria must be met. The first three criteria are met for *F. occidentalis*, however, the last criteron, freedom of choice of mate by female appears not clear enough to make a firm conclusion. As observed by Terry and Schneider (1993), females generally mate with the first male encountered and reject advances from other males over several days while some females enter an aggregation and reject all males. This suggests that females control the mating opportunities of the males but show little or no mate choice if they mate with the first male.

However, if the Höglund and Alatalo (1995) definition is applied then the mating system can be regarded as a lek mating system. Further work needs to be done to get a clearer picture of the mating system and whether there is female choice.

The adult males may be using 7-methyltricosane to enhance their attractiveness to females by winning fights and thus guide adult females in choosing a male for mating. However, this needs to be tested before a firm conclusion can be drawn.

8.2 Future directions

Based on the roles of the pheromones in the laboratory, it can be applied in the field to monitor and possibly control thrips. Aggregation pheromone attracts both sexes and activates them; this can be applied to get the thrips from their concealed position to open spaces whereby timely application of insecticides can be used.

El-Sayed *et al*. (2006) suggests mass trapping has potential to suppress or eradicate isolated populations of invasive pests at low densities. The aggregation pheromone may be

applied to traps in the field to attract both sexes thereby reducing the population of the thrips and consequently increase profitability. Another approach may be ‘attract and kill’ a situation where pheromone and insecticides are mixed together and sprayed directly to crop so that the thrips can come out of their hidden places and exposed to insecticides.

A test of the responses to the combination of the three major compounds is another exciting experiment that needs investigation to understand the complex nature of *F. occidentalis* behavioural responses. It is possible that behavioural response of *F. occidentalis* may be entirely changed with the mixtures in different concentration and ratios. This would provide valuable information whether the compounds are acting as a synergist or antagonist.

8.3 References

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